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Assessment of peanut allergen Ara h1 in processed foods using a SWCNTs-based nanobiosensor

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ABSTRACT

The goals of this research were to develop a rapid single-walled carbon nanotube (SWCNT)-based biosensor and to employ it to commercial food products for Ara h1 detection. The SWCNT-based biosensor was fabricated with SWCNTs immobilized with antibody (pAb) through hybridization of 1-pyrenebutanoic acid succinimidyl ester (1-PBASE) as a linker. The resistance difference (ΔR) was calculated by measuring linear sweep voltammetry (LSV) using a potentiostat. Resistance values increased as the concentration of Ara h1 increased over the range of 1 to 10^5 ng/L. The specific binding of anti-Ara h1 pAb to antigen including Ara h1 was confirmed by both indirect ELISA kit and biosensor assay. The biosensor was exposed to extracts prepared from commercial processed food containing peanuts, or no peanuts, and could successfully distinguish the peanut containing foods. In addition, the application of present biosensor approach documented the precise detection of Ara h1 concentrations in commercially available peanut containing foods.

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Single walled carbon nanotube; nanobiosensor; peanut allergy; Ara h1; peanut-process foods

Food allergy is one of the most common health concerns worldwide [1]. Sicherer et al. [2] reported that almost 5% of young children and 3–4% of adults suffer from food allergies in western societies. In general, food allergies arise as a result of immunological hypersensitivity, usually against certain proteins or glycoproteins (antigens) in food. There are a variety of symptoms that arise in allergic diseases; the common organ systems affected by a food induced allergic reaction include the gastrointestinal tract (diarrhea, emesis), the skin (atomic dermatitis, urticaria), and the respiratory system (asthma, rhinitis) [3]. Although virtually any food can cause allergy, a considerable proportion of food allergies is triggered by common sources, for example: milk, egg, peanuts, tree nuts, shellfish, fish, wheat, and soy [1]. Among them, the peanut is the leading source of most allergenic reactions. In 2008, the prevalent rate of peanut allergies in children was 1.4% in the USA; [2] the rates have been reported to be 0.2–0.4% and 0.3–0.75% in Denmark and France in 1989 and 1995, respectively [4]. Peanut allergy has been reported to occur at rates of 0.47% in 14–16-year-old Singapore school children, and 0.43% in 14–16-year-old Philippine school children [5]. Peanut allergens have been reported to be heat resistant and are often preserved in processed food containing peanuts, including cookies, chocolate, butter, crackers, and ice cream even after their production and processing [6]. Since peanut allergy is considered a life threating disease and accidental ingestion of peanut-containing foods cannot be ignored [7], the possible risks from allergy for unusual consumers can cause the shrinkage of the food industries in allergen labeling [8]. The common peanut allergens have been described in *Arachis hypogaea* and are referred to as *Arachis hypogaea* 1 (Ara h1) to Ara h13. Among them, Ara h1 is the most abundant peanut allergen. Ara h1 is a glycoprotein that belongs to the vicilin (7S) group and represents almost 12–16% of total peanut protein [9]. Between 35 and 95% of all allergic reactions are triggered in patients by Ara h1 [10]. The Ara h1 protein is resistant to digestion in the human gastrointestinal tract and is recognized by serum IgE in more than 90% of peanut-allergic patients [3]. Therefore, levels of Ara h1 can be considered to be a suitable biomarker in blood that can be used to identify the allergenicity of patients.

There is a great need for efficient methods to enable rapid detection of very low levels of peanut allergens. From the perspective of food safety, we aim to answer the needs of allergic consumers by developing such rapid and selective detection methods. Although enzyme-linked immunosorbent assays (ELISAs) have already proven to be effective tools for the detection of allergens, the process is difficult to perform, time

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consuming, not reusable, and is expensive [11]. The polymerase chain reaction (PCR) is a sensitive alternative method but is also not suited for rapid detection because it too is time-consuming, laborious, and costly [6]. Nano-biosensors are an attractive alternative choice for rapid and selective measurement of low doses of peanut allergen in real food samples. Furthermore, a biosensor is easy to use and does not have complicated sampling steps [12]. In addition, the biosensors can take advantage of the high specificity of diverse biological binding reactions such as antigen-antibody, enzyme-substrate, receptor-ligand, and other physical/chemical reactions in combination with a wide range of transducers. Therefore, the food industry is becoming more interested in biosensor technology owing to its potential for rapid, sensitive, simple, low cost, and portable detection. Although diverse biosensor methods have been reported for the detection of peanut allergens, including an optical biosensor [6], a screen printed based immunosensor [3], a stem loop DNA biosensor [9], and an electrochemical affinity-based sensor electrode [13], all of which have excellent detection limits and reproducibility, the majority of these assays are accompanied by technological difficulties and require highly trained personnel. Electrochemistry-based biosensors convert the interactions of the antibody with the targeted antigen into an electrical signal and therefore this could be very useful for the detection of peanut allergens [14].

Single-walled carbon nanotubes (SWCNTs) are hollow, cylindrical, molecular wires composed of a rolled graphite sheet [15]. SWCNTs have been studied as useful materials for the construction of analytical devices capable of detecting a range of biomolecules, including glucose, nucleic acids, and small proteins, in both prokaryotic and eukaryotic cells [16]. The integration of SWCNTs into the biosensor platform enhances the sensing performance and this is attributed to both its bio-compatibility and its size compatibility [16], as well as its structural flexibility [17], low capacitance, and axial electrical conductivity. Owing to its unique characteristics, the electrical properties of SWCNTs have been investigated in the interactions between nanoparticles and biomolecules. The fabrication of a SWCNT-based biosensor as an electrochemistry-based biosensor has been achieved using a field effect transistor design, in which either individual or networks of SWCNTs serve as electron channels between the source and drain electrodes [18]. In particular, SWCNTs and gold assembled onto the silicon wafer facilitate the electrical response and improve the sensitivity of the biosensor. The detection of these analyses using SWCNT-based chemiresistive/field-effect transistor (FET) sensors has been applied to medical sensor's in an *in vitro* system [19].

SWCNT-based biosensors generally have faster electron transfer kinetics than conventional carbon probes, advanced sensitivity properties, and a lower limit of detection [20]. They are well suited for providing cost-effective manufacturing due to the minimal cost of both CNTs and microelectrode sensor components. In addition, its surface biomolecules do not require any external bio-modification [21]. Although there have been only a few studies on the efficacy of SWCNT-based biosensors in detecting food-borne microorganisms such as *E. coli* K-12 [15] and *E. coli* O157:H7 [22], there have been no reports on their use for Ara h1 detection. In addition, there have been no previous reports on their application in commercial food products. This study is a continuation of a previous study aimed at developing a new SWCNT-based biosensor to detect Ara h1 and apply the biosensor to commercially produced processed foods that contain peanuts. The specific objectives of this research were to (1) develop a SWCNT-based biosensor immobilized with polyclonal anti-Ara h1 antibody capable of detecting Ara h1; (2) characterize the properties of the SWCNT-based biosensor; and (3) apply the SWCNT-based biosensor to detect Ara h1 present in real processed food products containing peanuts.

Materials and methods

Materials

SWCNTs were supplied by Chengdu Organic Chemicals Co. Ltd. (Chengdu, China) and their purity was greater than 95%. The peanut allergen Ara h1 (20000 ng/mL, naturally purified and prepared in 1% BSA/50% glycerol/PBS, pH 7.4) and the polyclonal antibody (pAb) against Ara h1 were procured from Indoor Biotechnologies Inc. (Cambridge, United Kingdom). The solutions used in the experiments were prepared using deionized water. Bovine serum albumin (BSA, 96–99%) was obtained from Baoman Bio-tech Co., Ltd. (Seoul, Korea). A 10% (v/v) phosphate buffer saline (PBS, 0.1 M, pH 7.4, 0.8% NaCl) was purchased from Life Technologies (Republic of Korea) and was prepared by mixing with purified water. Ara h1 was diluted for use in 10% PBS. All other chemicals were of analytical reagent grade, and were used without further purification. Triple distilled water was utilized throughout the experiments. Homogenization of solutions was achieved using a Vortex-Genie 2 (G560E).

Preparation of SWCNTs, linker and antibodies

A consistent SWCNT suspension was prepared by dispersing 0.1 mg of SWCNTs per milliliter of N, N-dimethylformamide (DMF) (Daejung Inc., Siheung, South Korea). Sonication of the purified SWCNT bundles was performed for 120 min using ultrasonic sonicator (UCP-02, AC-220 V, 60 Hz, 100 W) following procedures previously established in our laboratory. Afterward, 1-pyrenebutanoic acid, succinimidyl ester (1-PBASE) solution, as linker, was prepared by adding 9.8 mg of 1-PBASE into 5 mL of DMF [23]. The anti-Ara h1 antibody (pAb) was diluted one thousand-fold with 50 mM carbonate-bicarbonate buffered (pH 7.4). Following this, 80 µL of pAb, at a concentration of 1 mg/

mL, was applied to the 1-PBASE-immobilized SWCNT-based biosensor and stored at 4 °C in the refrigerator overnight for immobilization.

Manufacture of SWCNT-based nanobiosensor

The biosensor uses gold microelectrodes with 6.0 mm of gaps as the base sensing platform for signal processing. The electrodes were prepared by first depositing a 40 nm-thick layer onto the surface of the chrome (Cr)-deposited silicon wafer using an electron-beam evaporator (SRN-110-1505-R2, Sorona Inc., Pyeongtaek, South Korea) under high vacuum conditions (4.0×10^{-6} torr). The conductivity of the Cr-deposited silicon wafer was examined before gold (Au) deposition to ensure that there was no electrical current on the biosensor surface. The conductivity of the Au-deposited platform was also measured to confirm that the gold electrodes were able to function efficiently when each Au electrode was connected to an electrical tester.

The biosensor fabrication process included three steps: alignment, annealing, and functionalization of the SWCNTs [22]. The alignment process was conducted following the deposition of 15 µL of sonicated SWCNT suspension onto the junction in the gap between the Au electrodes. The aligned biosensor was then annealed by incubation in a drying oven at 80 °C for 15 min to obtain a good contact between the Au electrodes and the assembled SWCNTs. Following this, the annealed biosensor was washed with deionized (DI) water to remove unassembled SWCNTs from the sensor surface. For non-covalent functionalization, aliquots of 1-PBASE (15 µL) as linker solution were added to the electrodes containing the aligned SWCNTs. The sensor platform was then dried in the open air at room temperature for 2 h. After washing with DI water, biosensor device was covalently functionalized with the appropriate pAb (anti-Ara h1) onto the linker modified SWCNTs, and then incubated in a refrigerator at 4 °C overnight. A schematic diagram of the SWCNT-based biosensor is shown in Figure 1(A).

Detection of Ara h1 by the fabricated biosensor

The pAb immobilized biosensor was tested with 80 µL of diluted Ara h1 peanut protein solution at room temperature using a detection time of 30 min to allow for the antibody-antigen reaction to occur. After the reaction, the applied biosensor was washed with DI water to remove any unbound Ara h1 molecule from the sensor surface, and the resistance of the sensor electrode was measured using a potentiostat. A change in resistance confirmed the attachment of Ara h1 onto the anti-Ara h1 pAb and this change in resistance was only observed for Ara h1. In this study, linear sweep voltammetry (LSV) measurements were performed at each step using a potentiostat device (DY2013, EG Technology, Seoul, South Korea) at room temperature to measure resistance. The slope of the current/voltage (I/V) curves between 0.0 and 0.1 V for each treatment was estimated using LSV with linear regression analysis, and the resistance (Rs) was then calculated using the inverse of the current/voltage value. The resistance difference (ΔR), which is the main electrical response for the detection using the SWCNT-based nanobiosensor, was calculated using the following equation:

$$\Delta R = \left(R_1 - R_0\right)/R_0,$$

R_0 is the initial resistance measured by the potentiostat with only the linker (i.e. without Ara h1), whereas R_1 is the resistance measured with Ara h1. The experimental data are reported as means and the standard deviations were calculated based on the number of repeated experiments.

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were carried out to characterize the stepwise modifications of the electrode surface. A gold working electrode (2 mm diameter), Ag/AgCl (3.0 mol L^{-1} KCl) as a reference electrode and platinum wire as an counter electrode, which were performed in PBS buffer (pH 7.4) containing

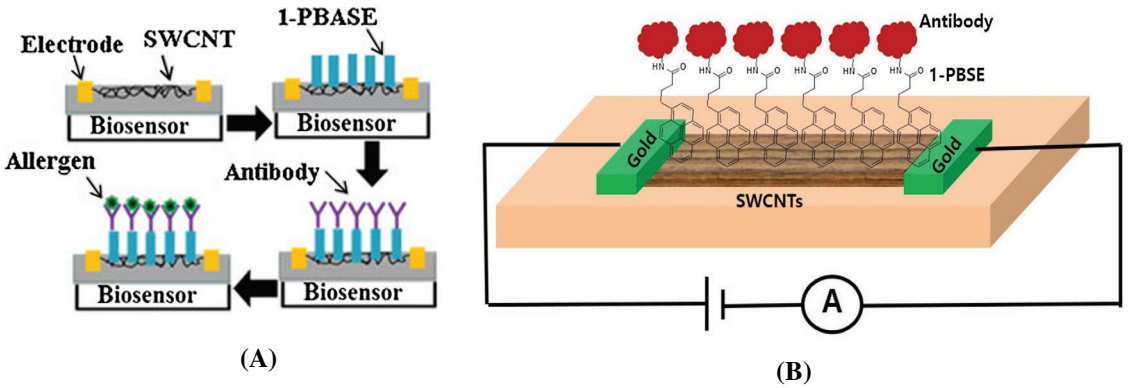

Figure 1. Scheme of SWCNT-based nanobiosensor for the detection of allergen-derived proteins in processed foods. (A) Manufacturing process used for the biosensor device. Schematic diagram showing the sensor device, in which the SWCNTs are modified with a 1-PBASE, and binding of the allergen to the polyclonal antibody produces resistance that can be mesasured; (B) Set up for biosensor measurement.

0.1 mol L^{-1} KCl and 2.5 mmol L^{-1} $[Fe(CN)_6]^{3-}$. The CV scan rate was 100 mVs^{-1}, while the potential fluctuated from +0.6 to −0.2 V. The frequency range for EIS ranged from 0.1 to 10^5 Hz at an amplitude of 0.01 V.

Evaluation of receptor sensitivity by indirect enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was performed following general procedures to determine the antibody specificity for Ara h1 detection. A 96-well microplate (SPL, Pocheon, South Korea) was incubated with 100 µL/well of serially diluted protein allergens at 37 °C for 1 h. After being washed three times with 200 µL PBS, unbound areas of the microplate were blocked with 200 µL/well of 1% bovine serum albumin (BSA) at room temperature for 1 h. Following this, the plates were washed again with 200 µL PBS, and 100 µL of the pAb Ara h1 solution (at a dilution of 1:1000) was added to the appropriate wells, and the plate incubated at room temperature for 2 h. After washing as above, 100 µL/well of an alkaline phosphatase secondary antibody (Sigma-Aldrich Inc., St. Louis, MO, USA), which was diluted to 1:1500, was added to each well and incubated at room temperature for 1 h and then the plates were washed again. p-nitrophenyl phosphate (KPL, Gaithersburg, MD, USA, 5 mg in 5 mL of 1 M ethanolamine buffer) was then added to each well as an alkaline phosphatase substrate. Following this, the optical density was measured using a micro-plate reader (Synergy H1 hybrid reader, Seoul, South Korea) at a wavelength of 405 nm. After a 30-min incubation in the dark at room temperature, color development was re-measured at 405 nm [24].

Difference of absorbance = Absorbance at 405 nm after 30 min − Absorbance at 405 nm at 0 min.

Transmission electron microscopy imaging

The nanostructures of the SWCNT and the Ara h1 molecules adsorbed onto the carbon nanotubes surfaces were observed using transmission electron microscopy (TEM) (Tecnai – 20, FEI, Korea University, South Korea) at 873 kV. The TEM images were obtained using thin films of a solution containing the SWCNT suspension with Ara h1 molecules in conjugation with anti-Ara h1 pAb deposited on a 400-mesh formvar carbon-coated copper grid. The sample preparation step for TEM was accomplished by dropping 2 µL solutions of the treated samples onto the TEM grids and wicking off the additional sample with filter paper after 40 s. Following this, the TEM grids were incubated at 60 °C for 4 h in a dry oven. The grids were then analyzed using TEM.

Sample preparation

Different processed food samples (peanut butter, peanut rice paper roll sauce, peanut chocolate, white bean milk, and chocolate milk), manufactured by Qingdao Jixing Foods Co. Ltd, South Korea, were bought from a local market and used to assess the applicability of the biosensor. Based on the product labels, peanut butter, peanut chocolate, and peanut rice paper roll sauce predominantly contained peanut proteins at 89.9, 15, and 21.6% respectively, along with sugar, vegetable fat, and phenolic compound, whereas, white bean milk and chocolate milk consisted of lentils, chick peas, butter bean, and oatmeal with grain mixture. In order to reduce the interference caused by high concentrations of phenolic compounds, and also to avoid the binding of the antibodies to non-target food components, a pretreatment process was required to achieve successful extraction of the allergen from the food samples. The semi-liquid peanut butter and peanut rice paper roll sauce were first sonicated for 10 min to disperse the food particles uniformly in the solution. In addition, peanut chocolate was first powdered and then 1 g of skimmed milk (Sigma-Aldrich, Germany) powder was added to bind to phenolic compounds in the chocolate. After that, each sample mixture was poured into 20 mL of Tris-HNO_3 (pH 8.2) buffer and heated at 60 °C with continuous mixing for 15 min [6]. Thereafter, the food samples were centrifuged at 2500 × *g* at 4 °C to produce a precipitate containing the bound phenolic compounds and a thick food sludge [6]. Following this the resulting supernatants were saved for biosensor and ELISA application.

Statistical analysis

The potentiostat measurements were repeated three times and the data were reported as average ± standard deviation. All analyses were performed using Microsoft Excel Software (Microsoft Excel Software 2013, Chicago, IL, USA), and the differences between the means were analyzed using Duncan with a defined significance level of $p < 0.05$.

Results and discussion

Manufacture of the SWCNT-based nanobiosensor

To investigate the electrochemical characteristics of the gold modified electrode, we hybridized the sensor with SWCNT, 1-PBASE as a linker, and an antibody as the receptor for the detection of the peanut allergen Ara h1, and analyzed the electrical properties (cyclic voltammetry, impedance, and resistance) (Figure 2).

As shown in Figure 2(A), when the gold electrode was assembled with bare SWCNT, a significant peak current was found. This indicates that SWCNTs immobilized on gold occupied a large surface area and were able to transfer electrons efficiently in the $[Fe(CN)_6]^{3-}$ solution. After immobilization of 1-PBASE as a linker on the SWCNT, the peak current decreased. After the sensing electrode surface was blocked with a pAb overnight and incubated with an Ara h1 containing solution, the peak current was

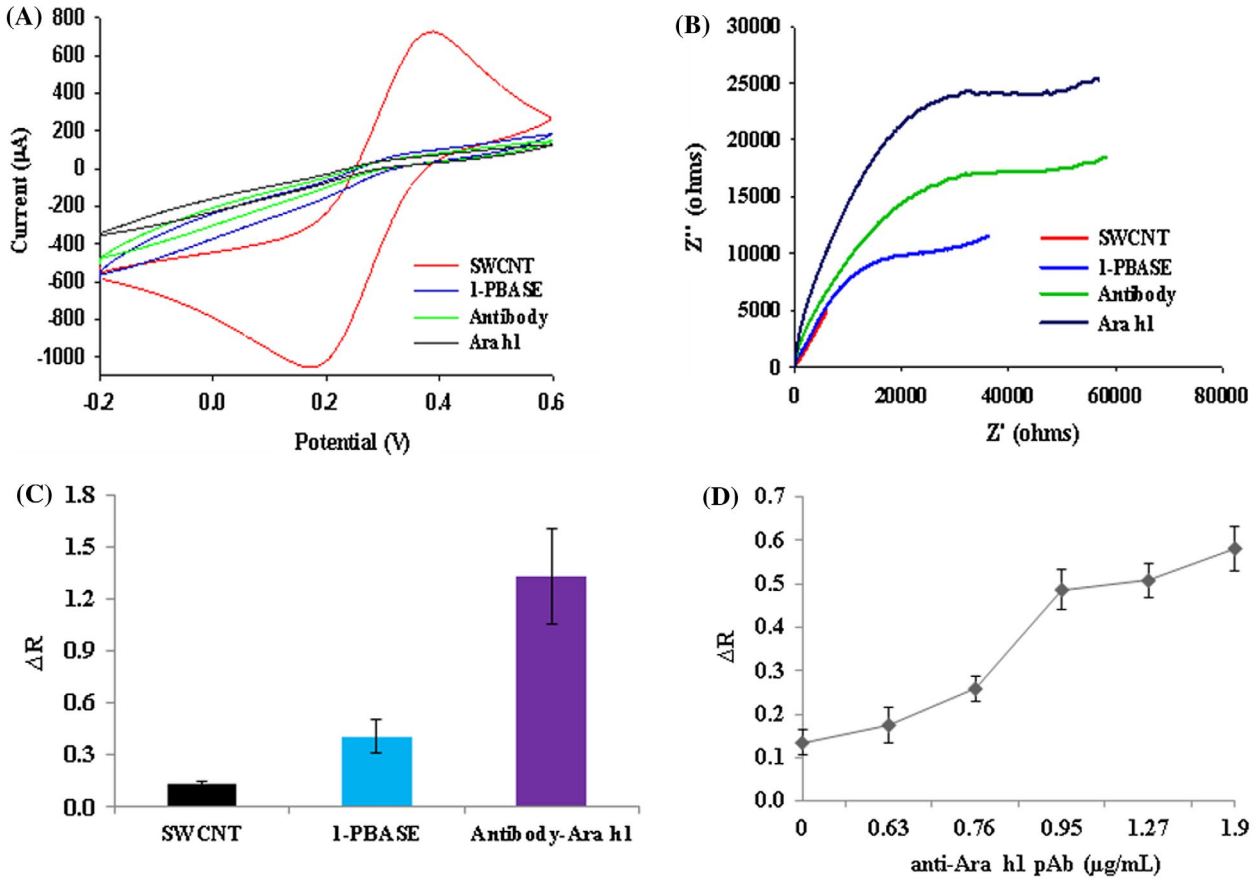

Figure 2. Optimal manufacturing process for the SWCNT-based nanobiosensor. Cyclic voltammogram (A) and impedance spectra (B) of SWCNT, 1-PBASE, antibody, and Ara h1; (C) Electrical resistance for SWCNT, 1-PBASE, and antibody-Ara h1; (D) Dose dependence of the electrical response (Δ*R*) in response to increasing levels of anti-Ara h1 antibodies. The experimental data are presented as the mean ± standard deviation ($n = 3$).

further reduced. These data indicate that the binding of pAb to the Ara h1 molecule inhibited the penetration of the redox species at the electrode surface, resulting in a decrease in voltammetry current behavior. The gold deposited sensor electrode was also characterized by electrochemical impedance spectroscopy (EIS) using SWCNT, the linker, and the antibody and the allergen Ara h1 in Figure 2(B). The impedance increased significantly after the biosensor electrode was immobilized with pAb and the Ara h1 solution, compared to the impedance of the bare SWCNT-modified electrode.

An previous study showed a similar phenomenon whereby adding a targeted nucleic acid to the sensor surface, caused a significant increase in impedance, whereas the peak current also decreased [25]. Several factors could play a role in the reduction of peak current and an increase in impedance. For example, the pyrenyl groups of the linker adsorb to the SWCNT through hydrophobic interactions, and the amine groups in the antibody directly react with the ester groups of the linker used to decrease steric hindrance [26]. In addition, the antibody and peanut allergen molecules are both proteins and in this case the electrons are located at the periphery of these proteins. Increased negative charges generated from the redox reaction are produced on the electrode surface and repel the negatively charged $Fe(CN)_6^{3-}$ anions in the redox solution, resulting in a decreasing in the voltammetry current and an increase in the EIS impedance [25].

Figure 2(C) shows the resistance response at different stages of assembly of the SWCNT-based nanobiosensor, SWCNT alone, SWCNT plus 1-PBASE as the linker, and SWCNT plus the pAb for Ara h1detection. The antibody was immobilized onto the SWCNT micro-bundle through a non-covalent interaction. The linker 1-PBASE was then used to bind the pAb antibody to the SWCNT through a covalent attachment. When the SWCNT bundle was modified with the linker and the antibody, the ΔR $((R - R_0)/R_0)$, the ratio of the resistance responses, where R and R_0 are the resistances after exposure to the buffer solution with and without the molecule, respectively, were increased to ca. 0.34 and ca. 1.23, respectively (Figure 2(C)). An increase in ΔR would be expected from the specific binding interaction between the antibody and the Ara h1 molecule. Aljaro et al. [22] reported that an increase in the resistance of the biosensor, i.e. a decrease in current, was attributed to the accumulation of negative charges derived from the antibodies. The binding specificity of the antigen and the antibody has also been claimed as being the reason for the prevention

of current flow, and consequently a substantial increase in resistance [27]. The developed biosensor is composed of a two-electrode system that has a simple structure which has several advantages including minimization, simplicity, and low cost of goods.

The antibody concentration in PBS buffer (pH 7.4) is one of the crucial factors in biosensor design that maximizes the binding efficiency of target molecules. The pAbs at various concentrations were immobilized on the functionalized biosensor template in order to determine the optimal concentration of antibody, using the increase in resistance as a readout of the amount of pAb immobilized on the surface of the SWCNT (Figure 2(D)).

The electrical response ΔR increased depending on the concentration of the anti-Ara h1 antibody, and in particular there was a significant increase in ΔR upon increasing the anti-Ara h1 antibody concentration to 0.95 µg/mL from 0.63 µg/mL. The ΔR increased further at antibody concentrations over 0.95 µg/mL because it seemed to derive unwanted immobilization process with the antibody/antibody reaction, not antigen/antibody reaction, in the bulk due to electrostatic repulsion force and steric hindrance. One possible reason for this is that antibody molecules have primary amines that will readily react with activated amine groups on other adjacent antibodies [3]. Such a reaction would likely result in less active sites available for antigen binding and this could lead to the creation of a peak in resistance for the sensor device. Therefore, the optimal anti-Ara h1 pAb concentration was determined to be 0.95 µg/mL for this SWCNT-based biosensor.

The nanostructure of the carbon nanotubes with pAb attached to Ara h1 molecules was observed using both transmission electron microscopy (TEM) and atomic force microscopy (AFM) to visually inspect the morphological structure of the SWCNT-based biosensor surface (Figure 3) [28].

Using TEM, Ara h1 immobilized on the SWCNT had a node shape, where the polypeptide chains of the Ara h1 protein was adsorbed on the hydrophobic surface via π–π stacking interactions. It should be noted 1-PBASE as the linker was mainly suspected for initiating the covalently surface binding and thought for activating controlled nanosize ring of nanotube-allergen molecules (Figure 3(A)) compared with the bare SWCNTs (inset of Figure 3(A)). The morphology of the biosensor surface was investigated using atomic force microscopy (AFM) analysis after immobilization of the bare SWCNT (inset of Figure 3(B)) or the SWCNT-pAb composite, as depicted in Figure 3(B). The surface roughness measurement (RMS) of the self-assembled SWCNT was found to be uniform and smooth, with a roughness of 14.9 nm (Table 1).

However, the surface roughness value increased to 78.2 nm, when 1-PABSE and the pAb were assembled onto the SWCNT layer. A higher magnification AFM image confirmed that carbon nanotube bundles were bound with pAb molecules by linker conjugation (Figure 3(B)). The increased RMS roughness is likely caused by the strong electronic coupling between the nanotubes and the pAb. The average RMS roughness values for both bare SWCNT and the pAb coated SWCNT were found to be significantly different. After immobilization of the pAb overnight, the surface was imaged using AFM and it was observed that the pAb molecules shielded the SWCNT sidewalls, resulting in a significant change in the average RMS roughness value from 11.0 to 47.5 nm respectively (Table 1). In addition, the 1-PBASE and pAb molecules on the sensor surface were not uniform in size and were roughly distributed around the SWCNT, as shown in Figure 3(B). Roughness on the biosensor surface was determined at 10 points and this analysis showed an RMS of 472.1 nm, compared to the bare SWCNT sensor surface, which had an average RMS of 116.1 nm, representing a fourfold increase in surface. It has been reported that carbon nanotubes and biological species on rough surfaces are much more densely populated, and their roughness values are significantly larger than those on smooth surfaces [29].

Sensor properties of the SWCNT-based nanobiosensor

The sensitivity and specificity of the biosensor was investigated using detection of the targeted peanut allergen

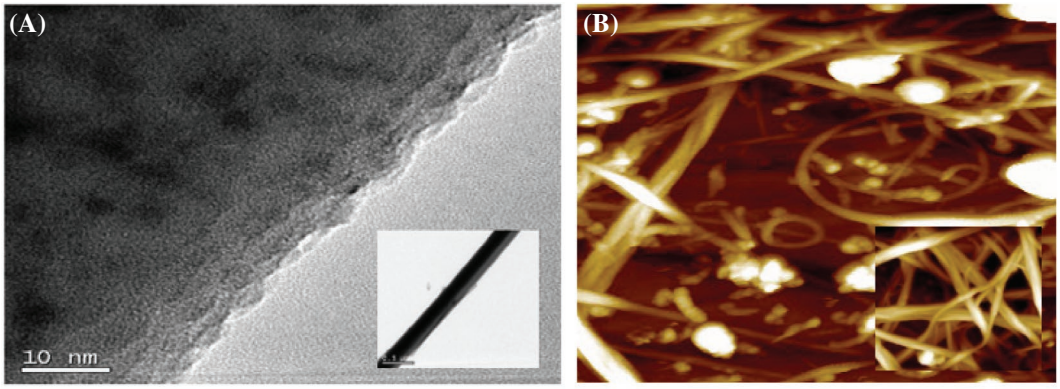

Figure 3. Image analysis of the SWCNT/1-PBASE/anti-Ara h1-modified device surface using (A) TEM and (B) AFM analysis. The insert image shows the bare SWCNT imaged by TEM and AFM.

Table 1. Analysis of the surface roughness of the assembly process in the SWCNT-based nanobiosensor using AFM.

Assembly process	RSM (nm)	Average (nm)	Average at ten points (nm)
SWCNT	14.9	11.0	116.1
SWCNT/pAb	78.2	47.5	472.1

(Ara h1) compared with an indirect ELISA test for the same antigen (Figure 4).

The sensitivity of the SWCNT-based nanobisensor was assessed at various concentrations of Ara h1 (0, 1.0, 10, 10^2, 10^3, 10^4, and 10^5 ng/L (Figure 4(A)). The SWCNT-based nanosensor was characterized electrically using the change in resistance calculated from the LSV data. ΔR increased depending on the concentration of Ara h1 in the PBS buffer, demostrating that Ara h1 molecules significantly decreased the electrical current of the sensor electrode. In the ELISA test, a signal could be detected at concentations above 10^3 ng/L Ara h1 (inset of Figure 4(A)). In addition, the time required to perform ELISA (approximately 6.0–7.0 h) was significantly higher than that for the biosensor assay (approximately 30 min).

The reason for the decreased current, i.e. increased resistance, likely arose as a consequence of the interaction between the pAb and Ara h1. In addition, charge

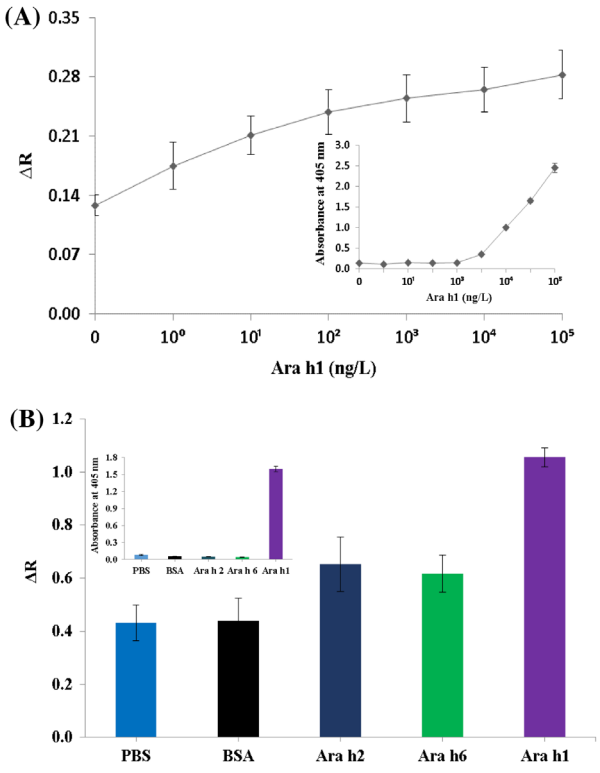

Figure 4. Sensor properties of the SWCNT-based nanobiosensor compared with the corresponding ELISA. (A) The sensitivity of the SWCNT-based nanobiosensor and the inset in (A) shows the sensitivity of the corresponding ELISA; (B) the specificity of the SWCNT-based nanobiosensor and the inset in (B) specificity of the corresponding ELISA test. The experimental results are presented as the mean ± standard deviation ($n = 3$).

transfer from hydrophobic amino acid residues located in the Ara h1 helical bundles or distortion of the SWCNT are also possible reasons for the increase in resistance [26,30]. It was also assumed that the increased amounts of Ara h1 antigen bound to the SWCNT-immobilized pAbs, as a result of the specific antibody-antigen interaction, increased the resistance of the p-typed SWCNTs in the debye length where the biosensor was considered as sensitive device [22].

The specificity of the biosensor is an essential characteristic for the practical application of this biosensor in specifically detecting peanut allergens [31]. The specificity of the SWCNT-based nanobiosensor was assessed using potentiostat signals derived from Ara h1 compared with the signals generated from other peanut allergen derived proteins (Ara h2, and Ara h6), as shown in Figure 4(B); the inset shows comparable data using the ELISA test. The concentration of each peanut allergen was kept constant at 10^3 ng/mL to ensure the consistency, while PBS was used as control. The biosensor was able to specifically detect Ara h1 compared to other peanut allergens ($p < 0.05$). The data indicate that the pAb-modified biosensor surface had a high affinity for the intended target protein. This result was in good agreement with the selectivity result obtained by Sun et al. [9] where a pAb assembled on a stem loop biosensor surface had a high selectivity for Ara h1.

The ELISA test showed a similar specificity to the biosensor assay. In the indirect ELISA test, only Ara h1 in combination with the anti-Ara h1 antibody, produced a high absorbance value (1.6). Other peanut allergens, including Ara h2 and Ara h6, produced absorbance values that were less than the cutoff value of 0.079 (mean of the negative control + 0.009) (inset of Figure 4(B)).

The ELISA test has been widely reported to be a specific assay using pAb, and for this reason it has been used for the consistent detection of Ara h1 in both PBS buffer and in supernatants derived from various food products. However, the ELISA test has disadvantages related to the high cost of the measurement instrument, a time-consuming procedure, and lack of portability, etc. Therefore, on the basis of this study, this SWCNT-based biosensor could be a useful alternative detection method in the area of food safety, that requires only a short detection time, is portable for measurement in the field, and has low cost, amongst other aspects.

Detection of Ara h1 in processed foods

The direct quantification of peanut allergens extracted in food sample supernatants is known to be difficult [6], so a measurement of the ΔR of the sensor electrode, with pure Ara h1 as the standard curve, was considered to be best option for peanut protein quantification. Although diverse biosensor methods have been reported for the detecting peanut allergen Ara h1 in real food extracts using constructed calibration curve, including

a screen printed based immunosensor [3], a stem loop DNA biosensor [9], and microfluidic sensor platform [13], according to our knowledge, there is no reports on efficacy of SWCNT-based biosensor for detecting Ara h1 in real food extracts. In addition, the SWCNT-based biosensor facilitated some advantages over the other assay, for example, advanced sensitivity properties, rapid electrochemical detection approach and higher selectivity. In order to estimate the amount of extracted Ara h1 in food supernatants, the ΔR from the SWCNT-based biosensor electrode was measured using food supernatants prepared from processed foods. ΔR value obtained was interpolated and compared with the standard curve (inset of Figure 5), and the concentrations of the extracted Ara h1 were calculated from linear part of the standard curve [32]. The concentrations of extracted Ara h1 measured in actual foods are shown in Table 2. The precision of this method was evaluated to determine biosensor performance using different professed foods and the obtained precision for the SWCNT-based biosensor was comparable to that seen with the commercial ELISA test. The biosensor showed significant precision for detection of Ara h1 in the food mixture.

The ΔR values were only significant in processed foods containing peanuts, whereas non-peanut containing foods produced a negligible biosensor response (Figure 5). The reason of undetectable biosensor signal might be non-specific bindings of pAb, confirmed that non-peanut containing processed food did not contain any of the peanut allergen Ara h1. This result was in good agreement with the results obtained from the ELISA test, where Ara h1 was detected only in processed foods that contained peanuts (Table 2). In addition, though, other food ingredients such as proteins, lipids and carbohydrates in the sample extracts was a major concern for interfering biosensor signal, this result could be a clue to clarify that electrochemical response of SWCNT-based biosensor could not be affected by their existence to detect Ara h1 as antigen in food extracts.

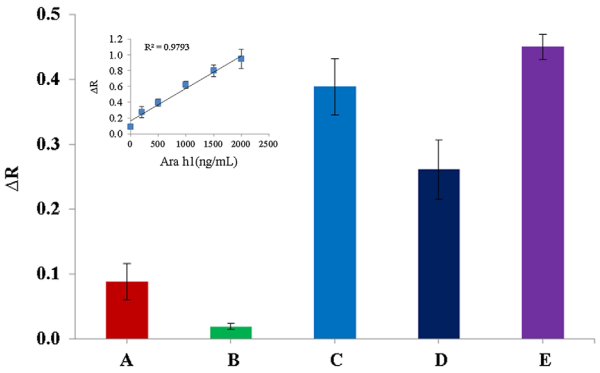

Figure 5. Real food test of the SWCNT-based nanobiosensor. The biosensor response to real foods for Ara h1 detection compared with the calibration curve. The experimental results are presented as mean ± standard deviation ($n = 3$). A represents chocolate milk; B represents white bean milk; C represents peanut rice paper roll sauce, D represents peanut chocolate, and E represents peanut butter.

Table 2. Detection of Ara h1 levels in real foods using the SWCNT-based nanobiosensor.

Food description	Ara h1 (ng/mL) SWCNT-based nanobiosensor	Indirect ELISA	Precision (%)
White bean milk	ND	ND	NA
Chocolate milk	ND	ND	NA
Peanut rice paper roll sauce	565.4 ± 77.7	518.2 ± 15.5	109.1
Peanut chocolate	246.5 ± 64.4	231.8 ± 4.9	106.3
Peanut butter	719.3± 309.6	706.9 ± 17.3	101.7

ND: Not detected; NA: Not applicable.

Conclusions

A functionalized SWCNT-based biosensor was developed for the label-free, rapid, and sensitive detection of the peanut allergen Ara h1. The biosensor has high performance, miniaturized structure, and sensitive response compared to other conventional methods. The immobilization of the anti-Ara h1 pAb at several concentrations on the sensing platform allowed for optimization of the sensor's signal responses. In addition, biosensor selectivity was confirmed towards Ara h1 ($p < 0.05$), whereas negligible changes in ΔR have been described for Ara h2 and Ara h6. The SWCNT-based biosensor also offered precise detection of Ara h1 in solutions prepared from processed foods containing peanuts. Importantly, the data showed that the SWCNT-based biosensor could be a promising approach for the detection of food allergens in processed foods containing peanuts, especially compared with the indirect ELISA test. Although the SWCNT-based biosensor is label-free, rapid, and can sensitively detect the target allergen, this study also suggests that a pretreatment extraction process is necessary when food samples are analyzed using the biosensor method. Therefore, future studies should explore methodolgies for the application of SWCNT-based biosensors without the need for sample extraction.

Author contributions

A. Sobhan carried out the experiment and wrote the manuscript with the support by J. Lee. J. H. Oh and M.K. Park advised the data analysis. S. W. Kim helped to supervise the project. J. Lee and C. Park conceived the original idea. J. Lee supervised the project.

Disclosure statement

No potential conflict of interest was reported by the authors.

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