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Electrical Immunosensor based on Dielectrophoretically-deposited Carbon Nanotubes for Detection of Influenza Virus H1N1

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Abstract

The influenza virus has received extensive attention due to the recent swine-origin H1N1 pandemics. This paper reports a label-free, highly sensitive and selective electrical immunosensor for detection of influenza virus H1N1 based on dielectrophoretically deposited single-walled carbon nanotubes (SWCNTs). COOH functionalized SWCNTs were deposited on a self-assembled monolayer of polyelectrolyte polydiallyldimethylammonium chloride (PDDA) between two gold electrodes by the dielectrophoretic and electrostatic forces, making reproducible, uniform, aligned, and aggregation-free SWCNT channels (2-10 µm length). Avidin was immobilized onto the PDDA-SWCNT channels and viral antibodies were immobilized using biotin-avidin coupling. The resistance of the channels increased with the binding of the influenza viruses to the antibodies. These immunosensors showed a linear behavior as the virus concentration was varied from 1 to $10^4$ PFU/ml along with a detection time of 30 min. The immunosensors with 2 µm channel length detected 1 PFU/ml of the influenza virus accurately ($R^2=0.99$) and selectively from MS2 bacteriophages. These immunosensors have the potential for a rapid clinical diagnosis component of a point-of-care test kit.

Keywords: Carbon nanotubes; influenza virus; H1N1; immunosensor; dielectrophoresis; PDDA; bacteriophages.
1. Introduction

Continuous outbreaks of highly pathogenic influenza virus H1N1 and many cases of human infection have caused significant international concern. Influenza viruses spread out easily by air transmission and the infection through the respiratory system is quickly done, resulting in an urgent need to detect the influenza virus rapidly and reliably.\(^1\) Conventional virus detection methods such as diagnostic test kits, enzyme-linked immunosorbent assay, and virus isolation and polymerase chain reaction are either poor in specificity, low in sensitivity, time consuming, expensive, or require a laboratory and a trained technician.\(^2-6\) Hence, it is highly desirable to develop a simple, sensitive, and inexpensive sensor to detect the virus rapidly and accurately.\(^7\)

Immunosensors are analytical devices that yield measurable signals in response to specific antigen–antibody interactions, thereby showing a quantity of the antigens in a sample. For virus detection, many immunosensors have been developed using electrochemical,\(^8-15\) frequency change,\(^16-18\) optical,\(^19-20\) and electrical\(^21-23\) properties. The electrical detection technique has several advantages such as simple and convenient measurements, which enables miniaturized and inexpensive biosensors. Therefore, it has a potential to revolutionize traditional laboratory techniques for virus detection.
Carbon nanotubes (CNTs) have proven to be a promising platform of ultrasensitive and miniaturized immunosensors for disease diagnosis because of superior mechanical and conductive properties such as high actuating stresses, low driving voltages, and high energy densities. However, the development of effective functionalization methods which can not only introduce homogeneous surface functional groups, but also cause less or no structural damage to CNTs remains a major challenge. Polydiallyldimethylammonium chloride (PDDA)-CNTs has been used in biosensing applications owing to its good film-forming ability and susceptibility to chemical modifications. The strong adsorption of the positively charged PDDA on CNTs may be due to the π-π interaction between PDDA and the basal plane of graphite of CNTs. This non-covalent polyelectrolyte functionalization cannot only lead to homogeneous surface functional groups on CNTs, but also preserves the intrinsic properties of CNTs without damaging their perfect surface structure. The uniformly distributed CNTs realized onto PDDA can offer much higher surface areas and electrocatalytic activity for virus detection.

Dielectrophoresis (DEP) has been considered one of the reliable, inexpensive, and efficient CNT deposition techniques and it involves the deposition of solution dispersed CNTs between electrodes with alignment by applying AC electric fields. Although chemical vapor deposition (CVD) is a common method for the direct growth of CNTs or a
network of CNTs, and CVD-grown CNTs have showed the best performance, the DEP technique is generally much simpler and more cost-effective, and does not require specialized materials and high temperature for the growth. Moreover, the alignment and the density of the deposited CNTs can be controlled by the AC frequency and the concentration of CNTs.\textsuperscript{28-30}

Here, we present a label-free and highly sensitive electrical immunosensor to detect influenza viruses H1N1 using the single-walled carbon nanotubes (SWCNTs) deposited on a PDDA self-assembled monolayer (SAM) by DEP. Avidin was immobilized on the SWCNTs, and viral antibodies were then immobilized using biotin-avidin coupling. The resistance shift of the SWCNT channels was measured as the concentration of the virus was varied from 1 to 10\textsuperscript{4} PFU/ml, and the selectivity of the immunosensor was also tested against high concentration MS2 bacteriophages.

In this study, the DEP technique was extended in conjunction with a PDDA SAM and the piranha treatment, which introduced abundant surface hydroxyl groups (–OH) via the hydroxylation process. That is, the SWCNTs were deposited by both the dielectrophoretic and electrostatic forces, which were exerted between the positively charged –NH\textsubscript{2} groups from PDDA and the negatively charged (–COOH– functionalized) SWCNTs, resulting in the reproducible, uniform, and aligned SWCNT deposition. This is
the significant difference compared to the previous DEP-deposited CNT sensors for virus
detection. García-Aljarado et al.²² developed CNT-based immunosensors for detection of
bacteriophage T7 without a SAM, yielding ~10 MΩ resistance, and the immunosensors
were annealed in order to have good contact. Lee et al.³⁰ presented CNT-based influenza
virus immunosensors by the layer-by-layer assembly method, which culminated in a
random, broken, and dense network of SWCNTs, where tube-tube junction may limit
charge transport.

2. Materials and methods

2.1. Biomolecules and chemical reagents

CNTs (90% of SWCNTs, diameter: 1–2 nm, length: 5–30 µm, COOH content:
~2.75 wt%) were purchased from M K Impex Corp. (Canada). PDDA (20 wt%, MW =
200–350k), avidin (A9275), bovine serum albumin (BSA) (A2153), N-(3-
Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) (03449), N-
Hydroxysuccinimide (NHS) (130672), glutaraldehyde (G765), osmium tetroxide (75632),
and isoamylacetate (112674) were obtained from Sigma–Aldrich (USA).
Dimethylformamide (DMF) (98%, D1021) and phosphate buffer saline (PBS) (1X, pH
7.4) containing 0.1% Tween 20 (P2006) were purchased from Biosesang Inc. (South
Korea). PBS (10X, pH 7.4, 70011-044) was purchased from Invitrogen Life Technologies (USA). Biotin conjugated mouse anti-influenza A monoclonal antibody (bs-1261M) was purchased from Gentaur molecular products (USA). Influenza virus H1N1 (KBPV-VR-33) was procured from the Bank of Pathogenic Viruses (South Korea). Bacteriophage MS2 (ATCC® 15597-B1™, \(1 \times 10^9\) PFU/ml) was procured from Koram Biogen Corp. (South Korea). Deionized water (dH\(_2\)O) (resistance: ~18.2 M\(\Omega\)) from the Millipore water purification system was utilized for preparation of the desired aqueous solutions (molecular biology grade). All the solutions and glassware were autoclaved prior to being used.

### 2.2. Microelectrode fabrication

A standard 6 inch silicon (Si) wafer with thermally grown silicon dioxide (SiO\(_2\), thickness: 500 nm) was cleaned with a piranha solution (\(\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2 = 2:1\)) at room temperature for 15 min, rinsed thoroughly with a copious amount of dH\(_2\)O, and dried with a nitrogen gas stream. Two electrodes for the source and the drain were patterned using photolithography, and then chromium (thickness: 20 nm) and gold (thickness: 200 nm) were electron beam evaporated sequentially onto the Si/SiO\(_2\) substrate. The source and the drain electrodes were tapered as shown in Fig. 1 to maximize the electric field at the edges.
and to increase the amount of uniformly distributed SWCNT connections during the DEP assembly. The gaps, which are referred to as channel lengths, between these two electrodes were 2 µm, 5 µm, and 10 µm and the width of the facing electrodes was 100 µm. The silicon wafer was then diced into chips (10 mm × 10 mm). The chips were cleaned in piranha solution followed by rinsing with dH₂O. These chips were then dried with nitrogen gas and either used immediately or stored under vacuum in a desiccator.

2.3. DEP assembly of SWCNT channels

Firstly, a SAM of PDDA was made as a precursor layer by pipetting onto the channel area for the charge enhancement and incubating for 15 min at 25°C prior to the DEP deposition step. A SWCNT suspension was prepared by suspending the SWCNTs in DMF (10 µg/ml) by sonication in a water bath for 90 min followed by centrifugation at 5,000 rpm for 1 h, and the supernatant liquid was discarded. The remaining SWCNT suspension can be kept for at least 1 month, and a 10 min sonication step is necessary before each use. The DEP assembly of the SWCNTs was performed by dropping 20 µl of the SWCNT suspension onto the channel area, and applying 10 V (peak to peak value) at 200 kHz for few sec. Finally, a thin film of uniformly distributed, parallelly aligned, and
aggregation-free SWCNTs was formed as a channel between the source and the drain electrodes. The chips were then rinsed with DMF, dH₂O followed by mild N₂ drying.

2.4. Immunofunctionalization and virus attachment

Firstly, 90 µl of avidin (1 mg/ml) was mixed with 5 µl of EDC (15 mM) and 5 µl of NHS (30 mM), and the mixture was incubated for 2 h at 25°C. Avidin was then immobilized onto the SWCNTs by depositing 10 µl of the mixture and incubating for 30 min. The SWCNT channels were washed with dH₂O and subjected to 10 µl of biotinylated monoclonal antibodies (10 µg/ml) specific to influenza virus H1N1 in an incubator at 37°C for 2 h. After washing with PBS (1X, pH 7.4), the SWCNT channels were incubated for 30 min in BSA (1 mg/ml) to prevent nonspecific binding followed by washing with PBS (1X, pH 7.4) containing 0.1% Tween 20 to remove loosely attached PDDA and SWCNTs. The immunosensors were then incubated in 10 µl of various concentrations of the viruses in PBS (1X, pH 7.4) for 30 min followed by rinsing, air-drying and resistance measurement. The influenza viruses were inactivated using Ultraviolet Crosslinker (CL-1000 UV crosslinker, UVP, Upland, CA, USA) prior to being used. A schematic of the developed SWCNT immunosensor and its test set-up are shown in Fig. 1. The selectivity of the immunosensor was assessed by incubating the immunosensor with high
concentration MS2 bacteriophages and measuring the resistance change due to the binding to the SWCNT channels.

2.5. Electrical measurements

The electrical measurement of the immunosensors was conducted by collecting the current-voltage (I–V) data from the immunosensors. The immunosensor response due to the virus attachment was measured as the normalized increase in resistance (NIR), \[ \Delta R = (R_{\text{Virus}} - R) / R, \]

where \( R_{\text{Virus}} \) is the resistance of the immunosensor after the exposure to the viruses and \( R \) is the immunosensor resistance after the antibody immobilization. The resistance of the immunosensors was measured after every functionalization step. The applied voltage was varied from \(-1.0\) to \(+1.0\) V, and current was recorded using a Source Meter® (2400, Keithley, Cleveland, OH).

2.6. Morphological and structural characterizations

Atomic force microscopy (AFM) and scanning electron microscopy (SEM) were used for morphological characterizations of the immunosensors. AFM images were obtained using a (Dimension) AFM 3100 (Veeco, USA). The chemical fixation was performed to image the influenza viruses captured on the SWCNT channel. First, the
immunosensors were immersed in 2.5% glutaraldehyde for 2 h and washed in 1X PBS buffer for 10 min. They were then immersed in 1% osmium tetroxide in distilled water for 2 h and washed again in 1X PBS buffer twice for 10 min each. The immunosensors were then placed in 25, 50, 70, 90, 100, and 100 % ethanol sequentially for 10 min each for dehydration. They were treated with a mixture of isoamylacetate and ethanol at a ratio of 1:3, 1:1, 1:0, and 1:0 sequentially for 10 min each for infiltration. The immunosensors were then placed in a critical point dryer (SPI Supplies) at 35°C at 1200 psi and coated with a thin layer of platinum for imaging with a scanning electron microscope (s-4800, Hitachi). A Fourier transform infrared (FT-IR) Varian 4100 (Agilent, USA) instrument was used for structural characterization without any further treatment of the samples (Supplementary data and supplementary Fig 1).

3. Results and Discussion

3.1. Morphological characterization - SEM

Fig. 2 a and b show SEM images of the uniformly distributed, aligned, and aggregation-free SWCNTs after the PDDA SAM and DEP assembly, demonstrating that the majority of the individual SWCNTs were reasonably aligned in parallel to the electrodes along with a few mis-aligned and tilted nanotubes. These aligned SWCNTs
contrast with a non-aligned and dense network of SWCNTs made by sedimentation (Fig. 2c). The PDDA SAM also helped the reproducibility of the deposition. This reproducible formation of uniform and aligned SWCNTs can be attributed to the combined effects of the DEP assembly and the SAM treatment. From these images, the average linear density of SWCNT arrays were estimated to be ~8 SWCNT/µm, which may strongly influence the performance of the fabricated SWCNT immunosensor.\textsuperscript{31} After the avidin and biotinylated antibody immobilization, uniform morphology appeared due to the interaction of avidin with the carboxyl functionalized SWCNT surfaces and biotinylated antibodies, revealing the successful immobilization of avidin and biotinylated antibodies onto the SWCNT surfaces (Fig. 2d). Fig. 2e shows a single influenza virus captured by the antibodies that were distributed on the SWCNTs, with several virus aggregates observed as well. The diameter of a single influenza virus was reported to be 80-120 nm,\textsuperscript{32} and previous studies have shown that the SWCNTs functionalized with specific antibodies were able to capture viruses.\textsuperscript{23}

3.2. Morphological characterization - AFM

Fig. 3 illustrates the schematic of the SWCNT deposition after the PDDA SAM formation along with the AFM micrographs of (a) PDDA, (b) PDDA-SWCNT, and (c)
PDDA-SWCNT-Avidin-biotinylated antibody. An initial monolayer of PDDA adsorbed was relatively smooth with surface roughness of 1.27 nm (Fig. 3 a). Fig. 3 b exhibits a layer of the DEP-deposited SWCNTs with an increased surface roughness of 80.2 nm. Fig. 3 c shows an AFM image after the antibody was covalently linked onto the SWCNTs using avidin-biotin coupling. The spiky nanotube features disappeared, and a globular surface generally reminiscent of thin antibody coatings was seen. It is clearly visible from Fig. 3 c that a remarkable decrease in surface roughness (15.4 nm) was a result of antibody attachment.

3.3. Resistance measurements and incubation time dependency

Fig. 4 a shows the resistance measurement of the immunosensors after PDDA-SWCNT deposition for various channel lengths of 2, 5 and 10 µm. The resistance was found to increase linearly with the channel length demonstrating a relationship of \( R = 9.97 \times L + 10.8 \), where \( R \) and \( L \) are expressed in kΩ and µm, respectively. The measurements also showed good repeatability, where the relative variations of the measurements were ~3%. García-Aljaro et al.\textsuperscript{22} presented ~10 MΩ resistance for DEP-deposited SWCNTs, and the chips were annealed in order to have good contact. We used PDDA as a precursor for the charge enhancement and achieved 1-100 kΩ for a thin film of uniformly distributed, aligned, and aggregation-free SWCNTs at 2-10 µm channel lengths. I-V
characteristics for the SWCNT immunosensors were shown in the supplementary data and supplementary Fig. 2.

The next experiment was focused on determining the required incubation time for maximum resistance change because the resistance can increase with the attachment of analytes. To this end, the SWCNT immunosensors were incubated in $10^4$ PFU/mL of influenza viruses at room temperature, and NIR was measured with incubation time. Fig. 4b shows that the NIR increased with incubation time up to 30 min attaining a plateau value of 1.5 with respect to the initial resistance. Accordingly, 30 min incubation time was used in the subsequent experiments.

3.4. Resistance measurements after each functionalization

The resistance measurements of the SWCNT immunosensors were performed after each step of the functionalization: PDDA-SWCNT, avidin, antibodies, and H1N1 virus ($10^2$ PFU/ml) (Fig. 5a). The antibodies immobilized on the avidin did not change the resistance as much as avidin did on the PDDA-SWCNTs. According to the measurements, there must be an electrostatic and/or structural change to induce observable resistance shift when avidin was immobilized on the SWCNT surfaces. This suggests that the resistance changes after the immobilization of influenza viruses were probably due
to their charge carrier donating/accepting property and/or the structural change of the SWCNTs due to the huge structure of the viruses. That is, the attachment of the viruses significantly influenced the electrical properties of the SWCNTs to increase resistivity.

### 3.5. Selectivity and sensitivity

To investigate the selectivity of the SWCNT immunosensors, MS2 bacteriophages with high concentration ($10^9$ PFU/ml) were used with 2, 5 and 10 µm channel length chips (Fig. 5 b). The averages and standard deviations were determined from 4 sets of immunoassay. The NIRs for MS2 bacteriophages were 0.093, 0.148, 0.188 for 2, 5 and 10 µm channel lengths, respectively, while the NIRs for influenza viruses ($10^2$ PFU/ml) were 0.899, 1.79, and 4.95 for 2, 5 and 10 µm channel lengths, respectively. The NIRs due to the MS2 bacteriophage attachment were 10-4% of those due to the influenza virus attachment even with much higher concentration of MS2 bacteriophages. This test showed that the SWCNT immunosensors were highly specific to influenza viruses against MS2 bacteriophages.

Fig. 6 shows the calibration plots of the immunosensors as the influenza virus concentration was varied from 1 to $10^4$ PFU/ml. The higher the influenza virus concentration, the larger the resistance shifts due to more influenza viruses adsorbed on
the channel surface. The immunosensor response, NIR, was a linear function of logarithm of viral concentrations between 1 and $10^4$ PFU/ml ($R^2 = 0.99$, 0.94, and 0.89 for 2, 5, and 10 µm channel lengths, respectively). The immunosensor linearity or accuracy decreased as the channel length increased. In fact, it was reported that longer channel chips showed larger measurement variations due to the attachment of other molecules in a virus solution on open binding sites. On the other hand, the longer SWCNT channel chips showed larger increase in the NIR with the increasing virus concentration, and they have larger sensing areas, both of which are favorable for a biosensing platform.

According to the measurements, the shortest channel immunosensor was the most precise and accurate. In fact, the NIR for highly concentrated MS2 bacteriophages was 0.093 while the NIR showed 0.17 for 1 PFU/ml of the influenza viruses. Moreover, the NIR for PBS buffer (1X, pH 7.4) showed 0.05. That is, this immunosensor can detect 1 PFU/ml of the influenza virus selectively from MS2 bacteriophages. This is highly sensitive considering that the range of influenza viral particles found in the infected swine nasal samples is $10^3 – 10^5$ TCID$_{50}$/ml (50% tissue culture infective dose) and a limit of detection of $\sim10^2$ TCID$_{50}$/ml was reported recently. Garcia-Aljarol showed a limit of detection of $10^3$ PFU/ml for bacteriophages T7 using SWCNT-based immunosensors.

Enhanced limit of detection in the present study may be attributed to uniformly distributed,
aligned, and aggregation-free SWCNTs. The reproducible generation of uniform and aligned SWCNTs on a PDDA SAM by the dielectrophoretic and electrostatic forces can have important implication for the large-scale fabrication of SWCNT-based biosensors.

4. Conclusions

In this study, we demonstrated a label-free, highly sensitive and selective electrical immunosensor to detect whole influenza viruses using dielectrophoretically deposited SWCNTs. The reproducible formation of a uniform, aligned, and aggregation-free SWCNT thin film between the source and the drain electrodes was observed on a PDDA SAM by applying both the dielectrophoretic and electrostatic forces, which showed an advantage over applying either as in previous studies. This immunosensor showed a linear behavior from 1 to $10^4$ PFU/ml along with a detection time of 30 min. The shortest channel (2 µm length) immunosensor can detect 1 PFU/ml of the influenza virus selectively from MS2 bacteriophages. This SWCNT-based electrical immunosensor has potential applications as a point-of-care test kit for rapid and simple clinical diagnosis or a component of a portable lab-on-a-chip system.

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References


Figure Captions:

**Fig. 1** Schematic illustration of the single walled carbon nanotube based immunosensor for H1N1 virus detection. The inset shows an optical image of dielectrophoretically deposited SWCNTs onto a PDDA SAM.

**Fig. 2** SEM images of (a) PDDA-SWCNT, (b) PDDA-SWCNT at higher magnification, (c) SWCNTs deposited by sedimentation, (d) H1N1 antibody immobilized PDDA-SWCNT, and (e) H1N1 antibody immobilized PDDA-SWCNT after capturing a single influenza virus.

**Fig. 3** Schematic of PDDA and SWCNT interaction along with representative AFM (tapping mode) images of (a) PDDA, (b) PDDA-SWCNT, and (c) H1N1 antibody immobilized PDDA-SWCNT.

**Fig. 4** (a) Resistance of the PDDA-SWCNT with various channel lengths of 2, 5, and 10 µm and constant width of 100 µm, and (b) Time-dependent response of the PDDA-SWCNT immunosensors after exposure to H1N1 virus ($10^4$ PFU/mL) with channel length of 2 µm and width 100 µm. The error bars indicate standard deviations from 4 sets of measurements.

**Fig. 5** (a) Surface binding studies of PDDA-SWCNT, avidin, biotinylated antibody, and H1N1 virus ($10^2$ PFU/ml) for various channel lengths of 2, 5, and 10 µm with constant width of 100 µm. (b) Selectivity tests of the SWCNT immunosensor against MS2 bacteriophages ($10^9$PFU/ml). The error bars indicate standard deviations from 4 sets of measurements.

**Fig. 6** Calibration plots of the SWCNT immunosensors showing that NIRs increased with the logarithm of the virus concentrations, where the channel length was 2, 5, and 10 µm. The error bars indicate standard deviations of 4 sets of measurements. X and Y represent the virus concentration and the NIR, respectively. The NIR showed 0.05 for PBS buffer (1X, pH 7.4).
Figure 1

- Bovine serum albumin (BSA)
- Biotin
- Avidin
- Antibody
- Virus

{Polydiallyldimethylammonium chloride (PDDA)}
Figure 2

(a) (b) (c) (d) (e)

82 nm Influenza virus
Figure 3
Figure 4

(a) Resistance (kΩ) vs. Channel length (µm)

(b) ∆R/R vs. Time (min)
Figure 5

(a) Resistance (kΩ) vs. Surface binding of H1N1 virus on PDDA-CNT, Avidin, Biotinyalted Ab.

(b) ΔR/R vs. Channel length (µm) for MS2 and H1N1 viruses.
Figure 6

The graph shows the relationship between the H1N1 Virus concentration (PFU/ml) and the relative change in resistance (ΔR/R) for different virus concentrations. The data is fitted with the following logarithmic equations:

1. \( Y = 0.7279 \ln(X) + 1.5296 \) with \( R^2 = 0.89 \)
2. \( Y = 0.2839 \ln(X) + 0.5468 \) with \( R^2 = 0.94 \)
3. \( Y = 0.1483 \ln(X) + 0.1736 \) with \( R^2 = 0.99 \)

The data points are represented for different virus concentrations and filter sizes, including 10 µm, 5 µm, 2 µm, and PBS.