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Flexible and disposable immunosensors based on layer-by-layer self-assembled carbon nanotubes and biomolecules

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ABSTRACT

A low-cost, flexible, and disposable immunosensor is presented in this paper. The single-walled carbon nanotubes (SWNTs) and biomolecules are self-assembled between two micro-patterned electrodes. The immuno-chip acts as a platform of a horseradish peroxidase (HRP) labeled sandwiched Enzyme-Linked ImmunoSorbent Assay (ELISA). The pH change induced by the biochemical reactions influences the electrical conductance of SWNT. A detection resolution of 0.4 ng/ml (2.5 pM) for normal rabbit immunoglobulin G (IgG) is demonstrated. The new fabrication technique and the HRP labeled detection protocol can be extended to the recognition of other antigens for critical applications to clinical diagnosis, food toxin detection, and environment monitoring.

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1. Introduction

Immunosensors transduce antigen–antibody interactions directly into physical signals, very suitable for applications including genomics, food toxin detection, and environmental monitoring. Immunosensors have been a main focus of extensive research on bimolecular recognition based on the capability of antibodies to bind various important antigens with high affinity and specificity.

Labeled and label-free immunosensor are the two main categories of the reported immunochips. Label-free immunosensors benefit from higher resolution, but rely on special process and suffer from poor yield and repeatability [1]. Currently, for most applications immunosensors use a label to increase the sensitivity of detection. Enzyme labels are very valuable because they can be used to efficiently catalyze the conversion of a substance into a detectable product, and the number of detectable molecules can be exponentially higher than the number of antigens. The most commonly observed form of detection is fluorescence due to its high sensitivity and stability. Although some efforts have been made to realize a compact and rapid detection system [2], fluorescence-labeled immunosensors are still too complex to be used. Electrochemical immunosensors have received much attention in on-site applications because they provide a simple, inexpensive, and accurate measurement of antigens based on either potential, current, capacitance, or conductance change caused by a specific bio-recognition reaction [3]. However, the current electrochemical immunosensors under investigation are somehow fragile, expensive or non-biocompatible due to the material nature and fabrication complexity.

Single-walled carbon nanotubes (SWNTs) are one of the most promising candidates for the development of immunosensors due to their unique and well-defined electrical and mechanical properties [4,5]. Yu et al. recently reported a sandwich assay on a CNT forest electrode, and such a system could detect prostate specific antigen (PSA) at a level of 4 pg/ml [6]. Cui et al. reported a electrochemical immunosensor using gold nanoparticles (GNPs)/carbon nanotubes (CNTs) hybrids platform with a horseradish peroxidase (HRP)-functionalized gold nanoparticle label for the sensitive detection of human IgG (HIgG) with a detection limit of 40 pg/ml [7], but the reported CNT based immunosensors are still suffered from expensive process and poor stability. In general, the electrical performance of the CNT films will drift with the microfluidic shock after the immersing of the CNT film in biological solutions.

To overcome the above hurdles, a low-cost, flexible, and disposable HRP-labeled immunosensor platform is presented [8]. A polymethylmethacrylate (PMMA) dielectric layer was coated on the surface of the self-assembled carbon nanotube composite film to shield the microfluidic shock and ion penetration in the substrate solution. The conductance change of the CNT film with different concentrations of target antigen is demonstrated.

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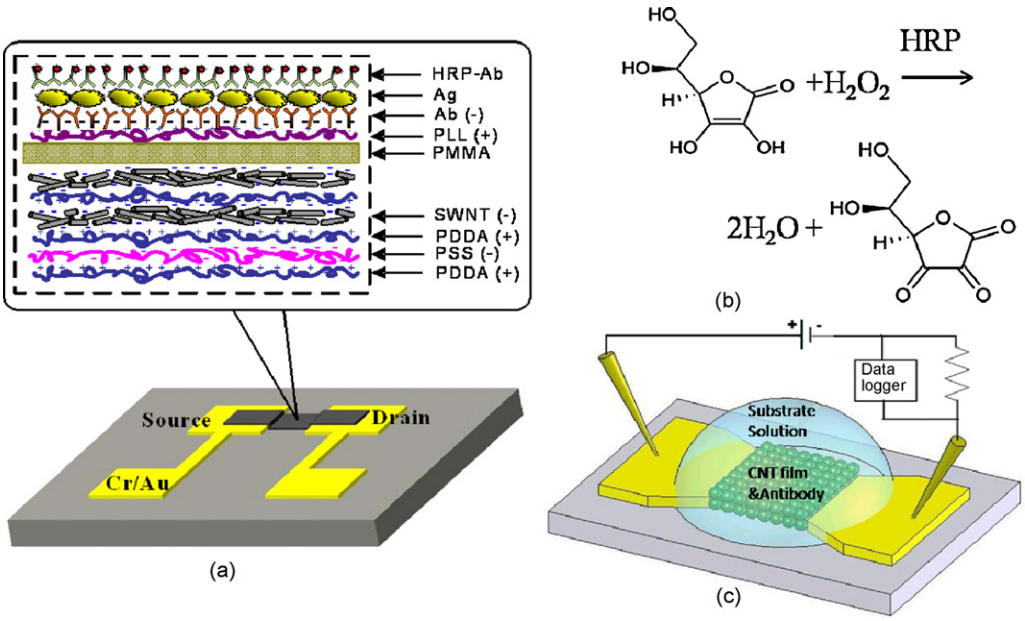

Fig. 1. (a) The structure of SWNT immunosensor, (b) HRP catalyzed ascorbic acid oxidization, and (c) testing apparatus.

2. Immunosensing principle

The self-assembled immunosensor platform in this paper is based on the principle of sandwiched Enzyme-Linked ImmunoSorbent Assay (ELISA) with an incorporation of new nano-materials into the structure design, as shown in Fig. 1(a).

The capturing antibody is immobilized on the solid support, and the antigen is added to specifically bind to the immobilized antibody. The labeled secondary antibody is allowed to bind with the antigen and to make the sandwich structure. Two types of antibody are required to have different epitopes on the antigen in order not to hinder the binding with each other. The secondary antibody used is a horseradish peroxidase (HRP) conjugated antibody. When 200 μl mixture of ascorbic acid and hydrogen peroxide (H_2O_2) was dipped on the surface of the chip (Fig. 1(c)), the ascorbic acid is oxidized to dehydroascorbic acid (Fig. 1(b)) by the catalytic behavior of HRP as shown in following reactions:

$$\mathrm{HRP} + \mathrm{H_2O_2} \rightarrow \mathrm{CompoundI} + \mathrm{H_2O} \quad \text{(i)}$$

$$2\mathrm{CompoundI} + \mathrm{AscorbicAcid} \rightarrow 2\mathrm{CompoundII} + \mathrm{DehydroascorbicAcid} \quad \text{(ii)}$$

$$2\mathrm{CompoundII} + \mathrm{AscorbicAcid} \rightarrow \mathrm{HRP} + \mathrm{H_2O} + \mathrm{DehydroascorbicAcid} \quad \text{(iii)}$$

Early study shows that the majority carriers in the layer-by-layer self-assembled SWNT films are holes because SWNT is a p-type material [5]. The structure, as shown in Fig. 1, is a p-type ion sensitive FET. The conductance in the SWNT channel will be

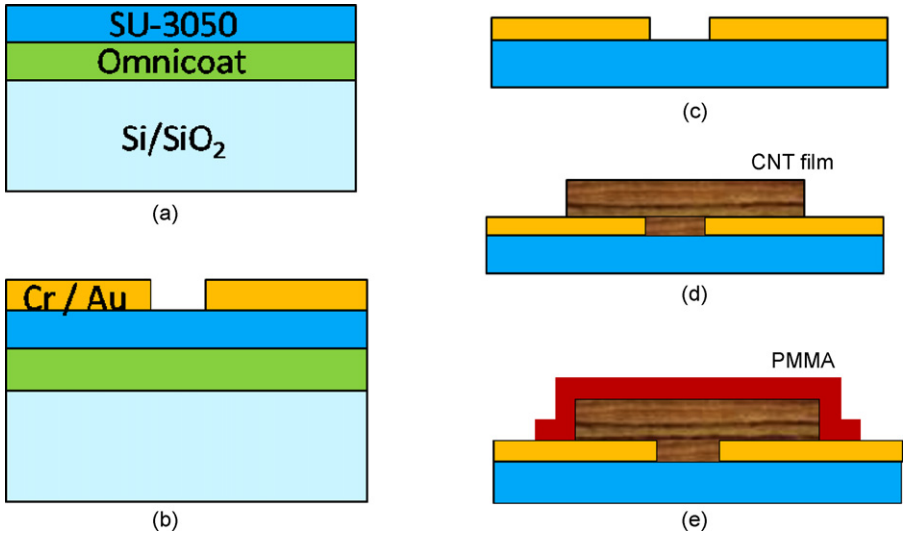

Fig. 2. The process flow of the biochip: (a) an Omnicoat layer and a SU8-3050 layer were spin-coated and UV exposed on a Si/SiO_2 wafer. (b) Cr/Au layers were deposited and patterned. (c) The wafer was immersed in developer overnight to peel off the SU8-3050 layer 150 μm thick. (d) Two bi-layers of (PDDA/PSS) and five bi-layers of (PDDA/SWNT) were self-assembled and patterned by lift-off process. (e) PMMA 300 nm thick was spun and patterned as a passivation layer.

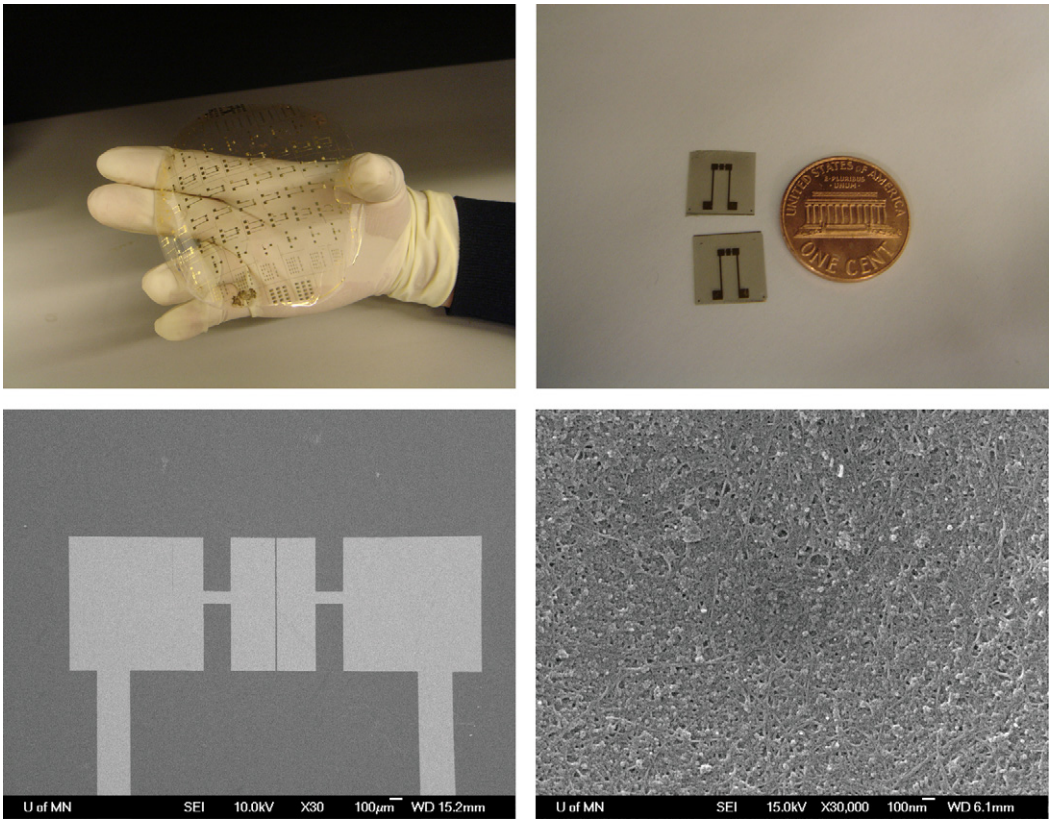

Fig. 3. Upper left: the optical image of immnuno-sensors on a flexible wafer; upper right: the optical image of individual immunochips; bottom left: the SEM image of source (S) and drain (D) electrodes of the immunochip. Bottom right: the SEM image of the surface of $(PDDA/PSS)_2$ and $(PDDA/SWNT)_5$ multilayer.

changed if some hydrogen ions aggregating on the top surface of PMMA, which is equivalent to applying a bias gate voltage on a p-type FET. During steps (ii) and (iii), the pH value of the solution increases, and the concentration of the hydrogen ions on the PMMA surface decreases, resulting in a conductance change of the underlying SWNT film. The change of the conductance was measured and recorded by a data logger.

3. Experiments

3.1. Materials

SWNTs (SZS002, Chengdu Alpha Nanotechnology Co., Ltd., China) were functionalized by 3:1 sulfuric acid and nitric acid to generate carboxylic groups, which facilitates the dispersion of SWNTs into water. The polyelectrolytes used in this study were poly(diallyldiamine chloride) (PDDA) and poly(styrene sulfonate) (PSS), which were from Sigma–Aldrich Inc. The aqueous PDDA and PSS had the concentrations of 1.5 and 0.3 wt%, respectively, with 0.5 M sodium chloride to enhance the surface properties. The normal rabbit immunoglobulin G (IgG) (sc-2027, molecular weight: 155 K) and goat anti-rabbit IgG (sc-3836) were received from Santa Cruz Biotechnology Inc. The HRP conjugated mouse anti-rabbit IgG (AP188P) was from Chemicon International Inc. Antibodies were diluted to 1× phosphate buffered saline (PBS, GIBCO 14190, Invitrogen Co., USA), so that the concentrations used were 400 ng/ml for capturing antibody and 40 ng/ml for detecting antibody. Ascorbic acid and o-phenylenediamine were provided by Sigma–Aldrich Inc.

3.2. Immunosensor fabrication

The immunosensor was fabricated using a technique combining "bottom-up" layer-by-layer self-assembly and "top-down" microfabrication. A layer of Omnicoat (3000 rpm, 30 s) and a layer of SU8-3050 (1000 rpm, 45 s, both from MicroChem Co., USA) were spin-coated on a Si/SiO_2 wafer, and exposed to UV ligth (or flood-exposed). Chromium/gold (Cr/Au) layers (100/200 nm thick) were deposited with e-beam evaporation, and patterned with UV lithography. Next, the wafer was immersed in developer (MF-319, Shipley) overnight to peel off the SU8-3050 layer 150 μm thick. Following that, photoresist (S1813, Shipley) 1.2 μm thick was patterned to protect two electrode pads for measurement. Two bi-layers of (PDDA/PSS) and 5 bilayers of (PDDA/SWNT) were self-assembled, followed by the lift-off process to expose the electrode pads. Finally PMMA (MicroChem Co.) 300 nm thick was spun and patterned as a passivation layer. Actually PMMA with different thickness (50 nm, 100 nm, 200 nm, and 300 nm thick) was coated and tested, and PMMA 300 nm thick was founded to be the minimum thickness to ensure a negligible leakage. The process flow was shown in Fig. 2, and the optical and scanning electron microscopy (SEM) images of the immunosensor are shown in Fig. 3. Here the self-assembled carbon nanotube composite film is dense, and the PMMA is not found to penetrate through the film. Experimentally a small conductance difference of the SWNT film was detected before and after the PMMA coating, which can be negligible to the sensor performance.

3.3. Surface treatment and fluorescence imaging

A glass slide was self-assembled with $(PDDA/PSS)_2(PDDA/SWNT)_5$ and coated with PMMA 300 nm thick similar to the above biosensor. Next, these samples was incubated in 400 ng/ml FITC conjugated donkey anti-rabbit IgG (sc-2090, Santa Cruz Biotechnology, Inc.), followed by fluorescence imaging. The following recipe was demonstrated to realize the best binding result: (1) etch with an oxygen plasma at a power of 100 W for 30 s, (2) immerse into

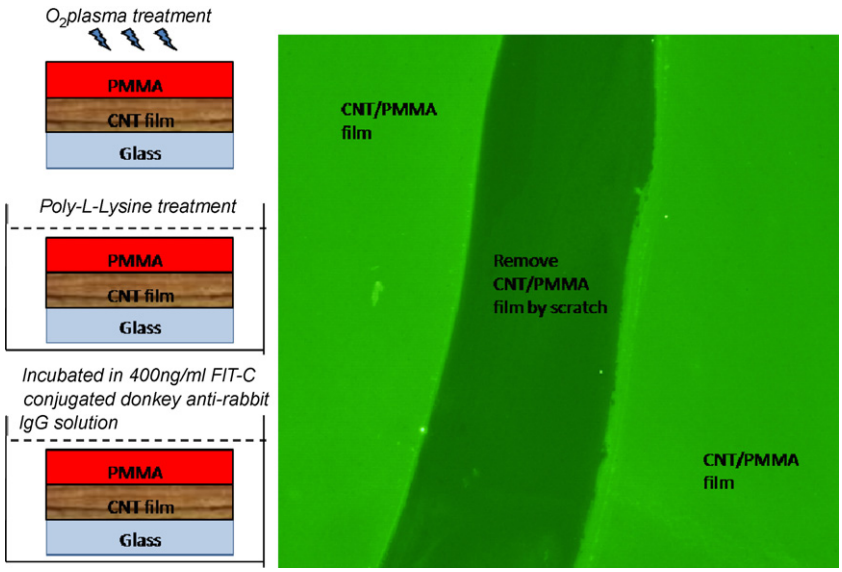

Fig. 4. The demonstration of the antibody affinity by surface treatment and florescence imaging.

0.1% PLL (poly-L-lysine, Sigma–Aldrich Inc.) aqueous solution for 1 h, drying with nitrogen stream, and (3) bake at 120 °C for 1 h. The florescence image was shown in Fig. 4, and some scratches were made on the slide to make a comparison.

3.4. pH sensitivity test apparatus and procedures

A standard buffer solution (200 μl at pH 5) was applied on the surface of the fabricated immunochips. A voltage of 1.0 V was applied to the immunochips, and a data logger was used to record the voltage and the current. After the current across the channel was stabilized, the conductance between the two electrodes was derived. The same chip was rinsed by DI water several times, and the test was conducted in buffer solutions at pH 6, 7, 8, and 9.

3.5. Immunoassay

The protocol of the immunoassay as well as the schematic of the sandwiched ELISA structure is illustrated in Fig. 5. The detailed procedure includes the following steps: (a) treat the surface of

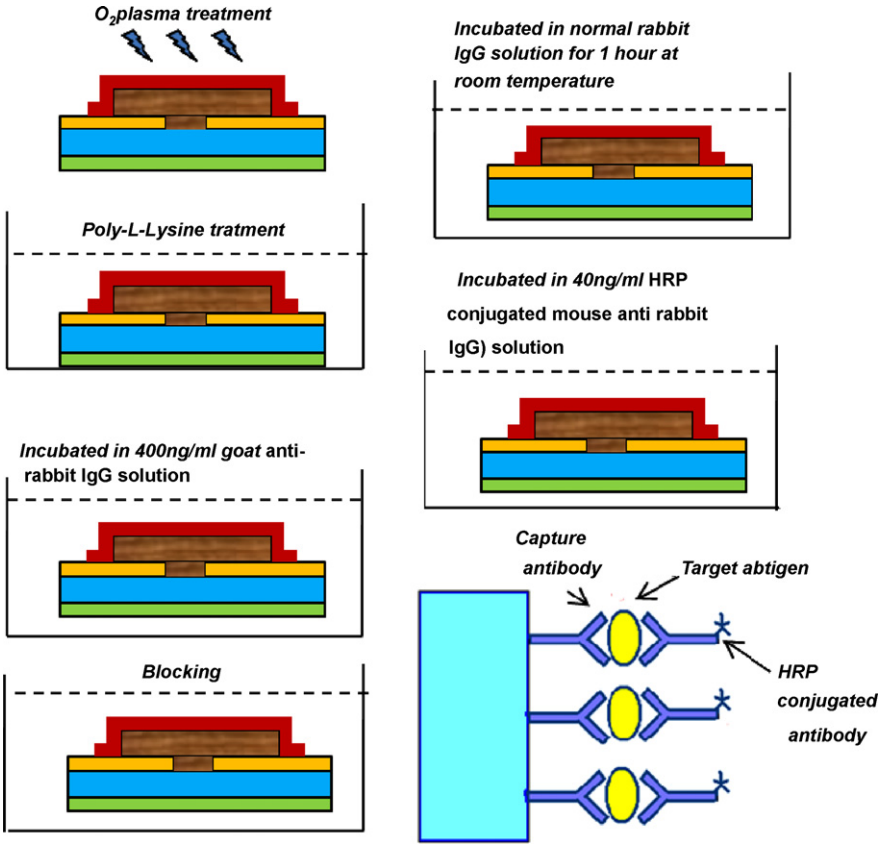

Fig. 5. The protocol of the immunoassay is listed from upper left to bottom right: the schematic of the sandwiched ELISA structure.

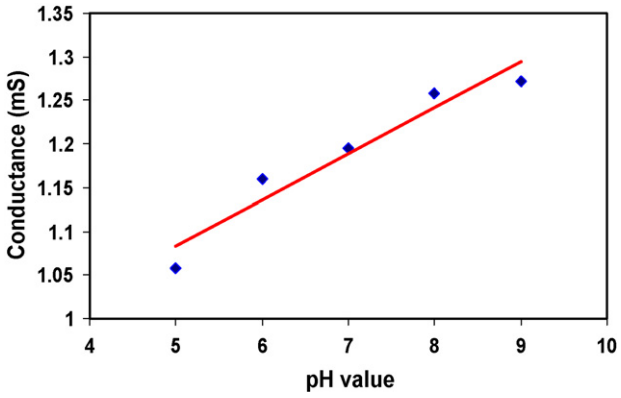

Fig. 6. pH-dependent conductance of the flexible SWNT immunosensor.

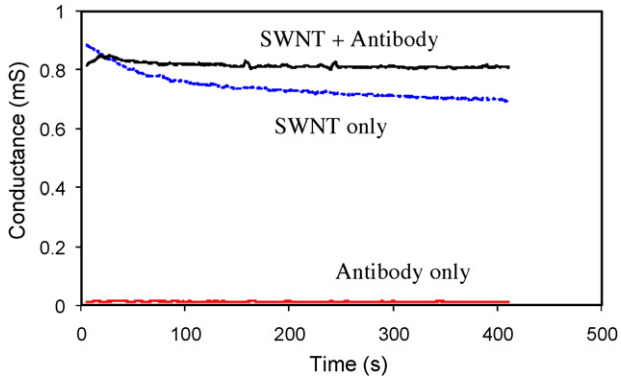

Fig. 7. The conductance changes of the devices with different coating. At the beginning, the devices were immersed into the substrate solution.

immunosensors with oxygen plasma at a power of 100 W for 30 s; (b) immerse into 0.1% PLL aqueous solution for 1 h, dry with nitrogen stream, and bake at 120 °C for 1 h; (c) incubate immunochips for overnight at 4 °C in goat anti-rabbit IgG solution at a concentration of 400 ng/ml; (d) rinse with PBS solution 3 times using a shaker (100 rpm, 19 mm circle) for 10 min each time; (e) immerse into 3% bovine serum albumin (BSA, Sigma–Aldrich Inc.) blocking solution for 3 h at room temperature and rinse as (d); (f) incubate for 1 h at room temperature in the target antigen (normal rabbit IgG) diluted into 1% BSA/PBS solution to make 0, 0.4, 4, 40, and

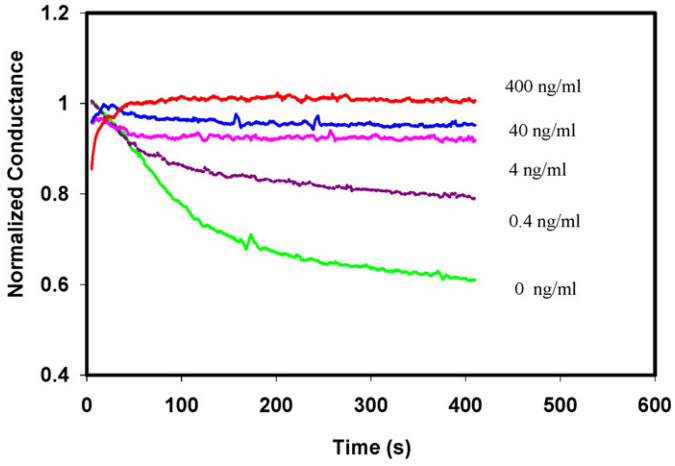

Fig. 8. The normalized conductance of the immunosensors at different antigen concentrations.

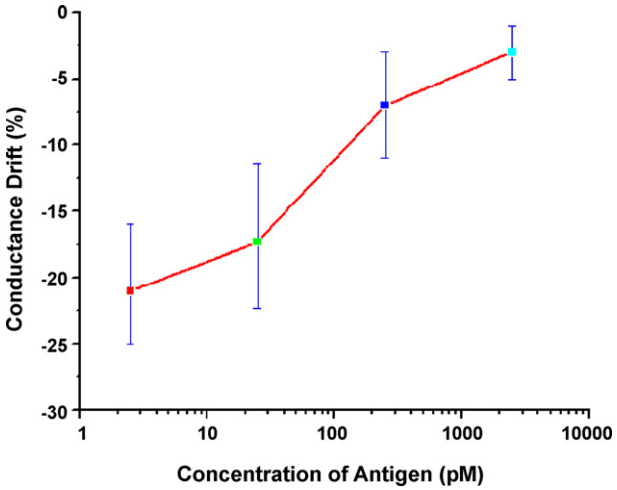

Fig. 9. The calibration curve of SWNT conductance drifts at different antigen concentrations.

400 ng/ml, respectively, followed by rinse as in step (d); (g) incubate for 1 h at room temperature in the detecting antibody (HRP conjugated mouse anti-rabbit IgG) solution diluted into 1% BSA/PBS solutions to be 40 ng/ml, followed by rinsing.

3.6. Immunosensing test apparatus and procedures

The mixture of 1.0 mM ascorbic acid and 1.0 mM o-phenylenediamine was produced by diluting to PB2 solution (1 mM Na-phosphate buffer, pH 6.0, with 15 mM NaCl). The role of o-phenylenediamine is to expedite the HRP catalyzed reactions. The substrate solution of 100 μl was applied on the surface of immunochips. It was observed that the drops can cover an area of about 1 cm × 0.5 cm on the sensor surface. Because the dimensions of the SWNT membrane on the immunosensor is 10 μm long, and 1000 μm wide, the solution was found to cover the SWNTs channel completely even after 20 min exposed at atmosphere in our experiment. Here the SWNT membrane was set to be apart from the surface of the solution drops, and surface tension or Maragori effect is found to be negligible to the measurement noise.

Next, 1.0 V voltage was applied, and a data logger was used to record the voltage and the current across two electrodes. After the current across the channel was stabilized, hydrogen peroxide solution (3.4 μl, a concentration of 0.3%) was carefully added into the substrate solution to minimize the shock. If HRP labeled antibody exists, dehydroascorbic acid would form. The conversion of ascorbic acid into dehydroascorbic acid during the enzymatically catalyzed H_2O_2 reduction causes a local pH shift, which is detected by the conductance change of the SWNT film.

4. Results and discussion

4.1. pH Dependence

Fig. 6 shows the relationship between conductance of the SWNT film and pH of the buffer solutions. The conductance increases with pH value. As a possible explanation, the hydrogen ion concentration decreases with the pH value which gradually changes from 5 to 9. This contributes to an equivalent negative bias applied to the gate dielectric layer of SWNT field-effect transistors. Martel et al. reported in 1998 that the carrier transport through SWNTs treated by the H_2SO_4/H_2O_2 solution is dominated by holes at room temperature, and it appears to be diffusive rather than ballistic [9]. Cui et al. found that the layer-by-layer self-assembled SWNT film treated by H_2SO_4/HNO_3 solution operated in the accumulation

mode with holes as the majority carriers, and behaved as a p-channel metal–oxide–semiconductor FET [5]. Therefore, the SWNT film is supposed to act as a p-type ion sensitive FET. A negative gate bias increases the density of holes in the underlying p-type carbon nanotube channel, thus increasing the conductance. This fact suggests that the SWNT film can play a role of electrochemical transducer, which converts pH value into electrical signal. Furthermore, the biochemical reactions that include pH changes can be characterized by the SWNT film.

4.2. Sensing capability

Fig. 7 shows the results of conductance measurement of a regular immunosensor, compared to the control devices without the SWNT film or without the immobilized antibody. The control device without the SWNT film had relatively low and constant conductivity although it was subjected to the substrate solution. Without the immobilized antibody, the conductance of the chip decreased since the surface pH value will change form about 7.4 (PBS solution) to below 5 in the ascorbic acid solution. Due to the pH increase in the HRP catalyzed reaction, the conductance of regular chips with the antibody and the SWNT film slightly decreased since the surface pH changes from 7.4 to about 6.0 in tens of minutes.

4.3. Detection range

Dozens of immunosensors were tested in different antigen solution with concentrations ranging from 0.4 to 400 ng/ml. The typical results are shown in Fig. 8, where the conductance of the SWNT film was normalized by its original stable value to reduce individual variation. As a result, the conductance of the SWNT film increases with the increasing antigen concentration.

The low concentration of antigen makes a small number of antigen molecules bound to capturing antibody, which enables a small number of HRP labeled antibody to bind to antigen. Therefore, a low concentration of antigen means a low concentration of HRP, and slows down the reaction of ascorbic acid oxidation. This creates a larger conductivity decrease of the SWNT film, resulting in a lower pH value of the substrate solution after a certain period.

The detection limitation is mainly determined by the non-specific bound HRP labeled antibody, which played a role of background noise in this immunoassay. To minimize the background signal from non-specific binding, we used the chips with antigen coating at zero concentration as the control chips. The valid results was recorded only when the control chips showed a reasonable conductance change which was significantly different from that of antigen coated chips.

Fig. 9 shows the calibration curve of the immunosensor. The detection limit of 0.4 ng/ml was demonstrated. We also tested the antigen concentration less than 0.4 ng/ml. However, the results were hardly identifiable from the control chips with 0 ng/ml concentration.

5. Conclusions

A flexible and disposable immunosensing device through the detection of conductance shifts of a self-assembled SWNT film was investigated. The sensor platform allows generic applicability by varying the antigen–antibody system developed on the flexible immunochips, and could be extended to the detection of many other biomolecules. Future improvements will be focused on higher sensitivity, lower detection limit, and integration with microfluidic detective systems.

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