Diagnosing ovarian cancer by identifying SCC-antigen on a multiwalled carbon nanotube-modified dielectrode sensor

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Abstract

Ovarian cancer starts in the ovaries in its earlier stages and then spreads to the pelvis, uterus, and abdominal region. The success of an ovarian cancer treatment depends on the stage of the cancer and the diagnostic system. Squamous cell carcinoma antigen (SCC-Ag) is one of the most efficient cancer biomarkers, and elevated levels of SCC-Ag in ovarian cancer cells have been used to identify ovarian cancer. Carbon is a potential material for biosensing applications due to its thermal, electrical, and physical properties. Multiwalled carbon nanotubes (MWCNTs) are carbon-based materials that can be used here to detect SCC-Ag. Anti-SCC-Ag antibody was immobilized on the amine-modified MWCNT dielectric sensing surface to detect SCC-Ag. The uniformity of the surface structure was measured with a 3D nanoprofiler, and the results confirmed the detection of SCC-Ag at ~80 pM. The specific detection of SCC-Ag was confirmed with two control proteins (factor IX and human serum albumin), and the system did not show biofouling. This experimental set-up with MWCNTs a dielectric sensing surface can lead to the detection of ovarian cancer in its initial stages.

Keywords: *cervical cancer, ovarian cancer biomarker, interdigitated electrode, multiwalled carbon nanotube surface, biosensor*

1. Introduction

Ovarian cancer is one of the most commonly occurring gynecological cancers. It primarily starts in the epithelium of the outer lining of the ovary [1]. Abnormal and uncontrolled cell growth in the ovaries causes cancer and eventually leads to a tumor. In general, the growth of ovarian cancer is separated into four stages. At the critical "stage I," only the ovary is involved, and with further stages, the disease moves into other areas, such as the uterus, pelvis, and abdominal region. Diagnosing ovarian cancer at this stage is a challenging issue, but detection helps to treat and destroy the disease. Early diagnosis of ovarian cancer with a suitable biomarker is necessary to save other organs before the disease starts spreading [1, 2]. Squamous cell carcinoma antigen (SCC-Ag) is an efficient cancer biomarker, and it has been found that elevated levels of SCC-Ag exist in people with ovarian cancer [3, 4]. A high sensitivity level of 73.3% was attained previously at "stage I," which helps anchor SCC-Ag's role in the screening process of gynecological cancer. Using histopathological samples, SCC-Ag in serum is a more sensitive biomarker for SCC tumors in ovarian serous

Abbreviations: *APTES, (3-Aminopropyl)triethoxysilane; EDC, N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride; IDE, interdigitated electrode; MWCNTs, multi-walled carbon nanotubes; NHS, N-hydroxysuccinimide; SCC, squamous cell carcinoma; SCC-Ag, squamous cell carcinoma antigen; UV, ultraviolet.*

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Received 30 May 2019; accepted 23 August 2019

DOI: 10.1002/bab.1808

Published online in Wiley Online Library (wileyonlinelibrary.com)

cystadenocarcinoma and is highly helpful in follow-up examinations of cervical cancer patients. Enhanced levels of SCC in serum have been considered for clinically detecting the recurrence of ovarian cancer [5]. This research is focused on quantifying the level of SCC-Ag on a multiwalled carbon nanotube (MWCNT)-modified dielectric sensor to diagnose ovarian cancer.

Carbon-based materials are attractive in the field of biosensor research due to their unique chemical, physical, and electrical properties [6]. In the past, various sensing methods with graphene have been proven to efficiently detect diseases. Several methods have been used to immobilize biomolecules, including DNA, RNA, antibodies, proteins, peptides, and aptamers, on graphene surfaces for biosensor applications [7–13]. MWCNTs are graphene-based materials that are usually hollow with cylindrical allotropes. They have a large surface area, high conductivity of electricity, and fast electron transfer, and are biocompatible [14]. Due to these positive features, MWCNTs are considered to be a valuable material in the field of biosensor research. Especially in electrochemical sensors, it has been proven that to increase the electrochemical activity of various biomolecules, graphene can be used to obtain enhanced electron transfer. In addition, biofunctionalization on MWCNT surfaces can be easily carried out due to its physical and chemical adsorption characteristics [15]. In this current study, the interdigitated dielectrode (IDE) surface was chemically modified with MWCNTs for chemical functionalization; the surface was further immobilized with an antibody for SCC-Ag interactions to detect ovarian cancer with voltammetry measurements.

2. Materials and Methods

2.1. Reagents and biomolecules

SCC-Ag was purchased from RANDOX Life Sciences (Antrim, UK), Malaysia. Anti-SCC-Ag antibody was obtained from Next Gene (Selangor, Malaysia). (3-Aminopropyl)triethoxysilane (APTES), ethanolamine, ethanol, phosphate-buffered saline (PBS, pH 7.4), human serum albumin (HSA) and MWCNTs were purchased from Sigma–Aldrich (Missouri, USA). Human blood clotting factor IX was acquired from Abcam (Cambridge, UK). N-Hydroxysuccinimide (NHS) and N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were procured from GE Healthcare (Illinois, USA). All chemicals and reagents were stored according to the manufacturers' recommendations.

2.2. Fabrication of IDE sensing surface

A silver IDE electrode was deposited on a silicon wafer sample <100> using a traditional wet etching method. Positive Photo Resist was coated on the silicon wafer, followed by soft-baking for 90 Dec. Ultraviolet light exposure was conducted for 10 Sec to allow the pattern to transfer from the IDE mask onto the surface of the samples. After that, the development process was carried for 15 Sec using an RD-6 developer. The sample was baked at 110 °C to remove unwanted moisture and to improve adhesion between the silver and SiO_2 layers. Finally, the unexposed area was removed using silver etchant for 23 Sec and then cleaned with acetone. Using this basic preparation, a further modification was performed using the chemical linker. The 3D nanoprofiler was used to analyze the surface of the dielectrode IDE.

2.3. Preparation of the MWCNT-modified IDE surface

The MWCNTs were washed thoroughly and treated with acid (a mixture of nitric acid (60%) and sulfuric acid (15%) in a 1:3 ratio). To prepare the surface-modified MWCNTs, the surface was first chemically functionalized into amine-groups by a silane reaction using APTES. A 3% solution of APTES was diluted in 30% ethanol and added to the IDE surface and kept overnight at room temperature (RT). The surface was then washed several times with 30% ethanol to remove the unbound APTES. To immobilize the MWCNTs on the APTES-modified surfaces, 0.1 mg of MWCNTs was suspended in dimethylformamide (1 mL) and the mixture was ultra-sonicated (4 H) in order to get a thorough dispersion before being placed on the aminated surface. This surface was further immobilized with 200 nM of SCC-Ag antibody, which was diluted in a PBS buffer and added on the NHS (50 mM) and EDC (200 mM) activated MWCNTs surface at a ratio of 1:1. After the probe attachment, the remaining surface was blocked with 1 M of ethanolamine to detect the SCC-Ag. The surface was washed thoroughly five times with a PBS buffer in between each immobilization, and the current changes were recorded after each immobilization.

2.4. Detection of SCC-Ag on the antibody-immobilized MWCNT surface

As described above, SCC-Ag was detected on the SCC-Ag antibody-immobilized MWCNT surfaces. After the surface was covered by ethanolamine, a concentration of 1 nM SCC-Ag was dropped on the surface and left for 15 Min to interact with the antibody and SCC-Ag. After that, the surface was washed with a PBS buffer and then the current changes were recorded for comparison. To check the limit of detection, different concentrations of SCC-Ag ranging from lower to higher (60 pM to 1 nM) were dropped on the SCC-Ag antibody-modified surfaces and the current changes were recorded for each experiment to evaluate the sensitivity.

2.5. Specific detection of SCC-Ag

After checking the limit of detection, specificity experiments were carried out by comparing two closely related proteins as controls (Factor IX and HSA): human blood coagulating factor IX and HSA. These two control proteins and the specific SCC-Ag were passed on to the SCC-Ag antibody-modified surfaces independently at a 1 nM concentration, and changes in the current level were monitored for the comparison.

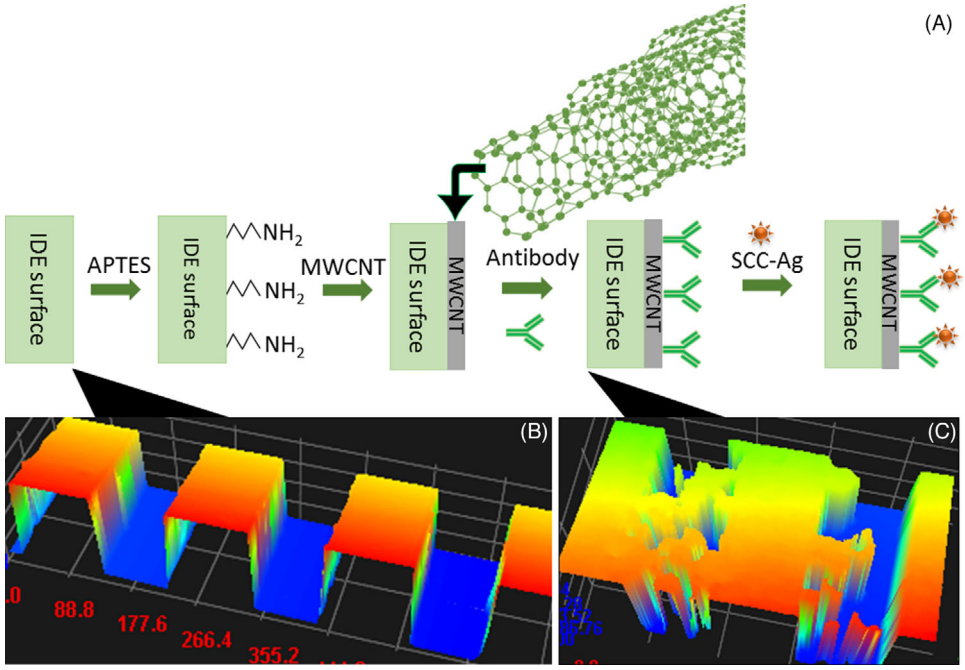

FIG. 1 *Analysis of the IDE surface. (A) Schematic representation of SCC-Ag detetction on a MWCNT-modified IDE surface. MWCNTs attached to the amine-modified IDE surfaces, and the antibody was immobilized. SCC-Ag was detetcted on the antibody-modiifed surfaces. (B) Surface morphology analysis on the IDE using a 3D nanoprofiler. The fingers and gaps on the surface are clearly shown. (C) The molecular attachment on the gap regions was confirmed using the 3D nanoprofiler. The attached molecules are seen clearly.*

3. Results and Discussion

Diagnosing ovarian cancer in its initial stages helps to treat it and keep it from spreading to other organs in the reproductive system. It has been widely accepted that SCC-Ag is a suitable biomarker for gynecological cancers, including ovarian cancer. It has been proven that an increased level of SCC-Ag exists in ovarian cancer patients. Detecting and quantifying the level of SCC-Ag is necessary for predicting the stages of this cancer. In this work, carbon-based, MWCNTs were modified onto an IDE sensor to identity SCC-Ag at low levels. MWCNTs show a higher resistance to current changes when biological interactions take place on their surface, and also they are highly biocompatible and available at low costs [16]. Highly efficient detection of glucose on MWCNT-modified surfaces has been demonstrated relative to single-walled carbon nanotube-modified surfaces. In addition, a four-fold higher current change has been noticed on MWCNTs compared with graphene [17–19]. By utilizing these positive features, an IDE surface has here been modified into MWCNT surfaces through amine functionalization to quantify the level of SCC-Ag. Figure 1A shows a schematic illustration of SCC-Ag detection on MWCNT-modified surfaces.

3.1. Surface morphology profile and functionalization

The fabricated sensing surface has finger and gap regions made up of aluminum dielectrodes on the silica surface. The silica surface was coated with zinc oxide to facilitate the chemical functionalization. Prior to the analysis, the surface was thoroughly washed by using 1 M potassium hydroxide to teather the surface with higher hydroxyl groups. Figures 1B and 1C are surface observations using a 3D nanoprofiler with the fabricated surface before and after the surface modifications, respectively. Figure 1B clearly demonstrates the presence of uniform fingers and gaps. Further, the distances between the fingers are found to be well aligned. With this confirmation, further surface modifications and functionalization were performed. First, the surface was modified into an amine, and then acid-washed MWCNTs were attached to this amine-modified surface. The immobilization of MWCNTs is due to the amine group created by APTES attaching to the functional groups on the MWCNTs. After that, via EDC-NHS activation, the antibody for SCC-Ag was immobilized on the MWCNT surface. The remaining surfaces were blocked by ethanolamine, and finally, the probe-modified surface was used to detect SCC-Ag.

3.2. Antibody immobilization on MWCNT-modified IDE surfaces

As explained previously, the antibody probe was immobilized on the MWCNT-modified surfaces through chemical conjugation in which the carboxyl group on the MWCNT-modified surface reacted with the amine group on the antibody. Figure 2A shows the current changes for each immobilized biomolecule. None of the various biomolecules had a current at $2.66E^{-08}$. After adding the ethanolamine, the current increased to $1.04E^{-08}$, which indicates the proper attachment of the APTES on the IDE surface. After that, when MWCNTs were allowed to bind on the

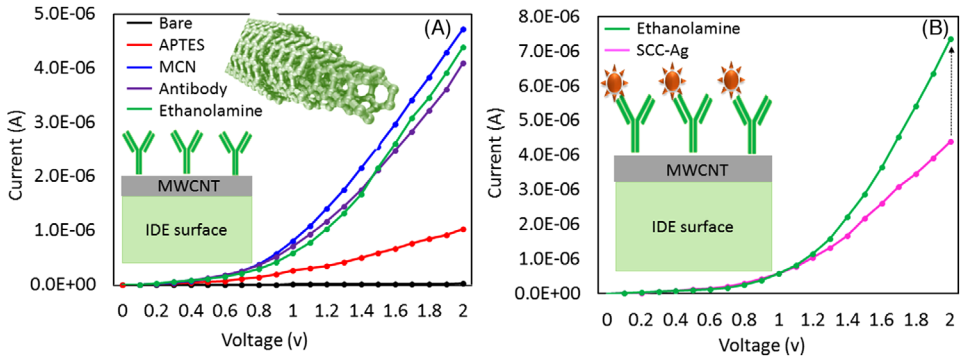

FIG. 2 *Surface modification of the IDE surface. (A) Antibody immobilization on the MWCNT surface. Current changes are clearly shown after each step is performed. Antibodies are immobilized as the probe shows the clear current increment on the MWCNT surface. (B) 1 nM of SCC-Ag detection on the antibody modified-MWCNT surface. After adding SCC-Ag the current level increased.*

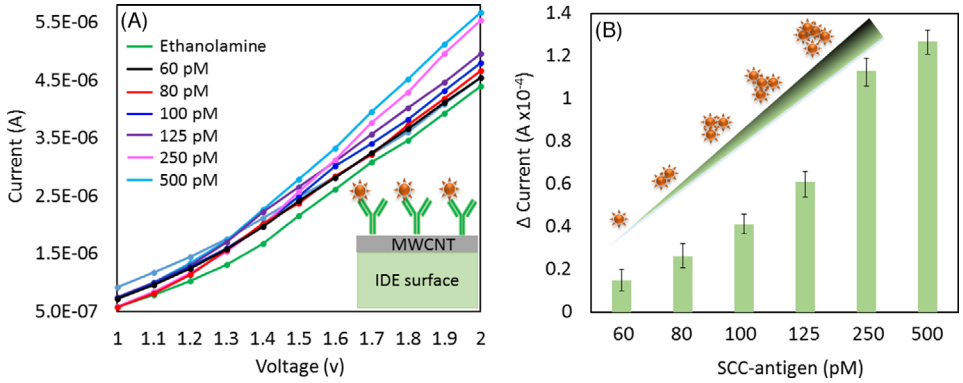

FIG. 3 *Dose-dependent interactions. (A) Detetcion of SCC-Ag on MWCNT surfaces. Different concentrations of SCC-Ag (60–500 pM) interacted independently on the antibody-immobilized surfaces. With increasing concentrations of SCC-Ag, the current levels also increased. (B) Current increment levels for each SCC-Ag concentration.*

APTES-modified surface, the current increased to $4.72E^{-08}$. This is almost 4.5 times the level of the APTES. This large current increase is due to the high charge changes on the surface after attaching the MWCNTs. Next, the anti-SCC-Ag antibody was immobilized through the EDC-NHS activation. It was found that the current level decreased to $4.10E^{-08}$, which might be due to the specific charge of the antibody. Finally, 1 M of ethanolamine was added to cover the remaining surfaces to avoid biofouling. In this case, the current increased to $4.4\ E^{-08}$. The overall current changes for each immobilization confirmed the proper immobilization of molecules on the MWCNT-modified IDE sensing surface. Figure 1C displays the molecular attachments after the blocking step; it is obvious that the surface modifications occurred with the above biomolecular immobilization.

3.3. Diagnosing dose-dependent SCC-Ag on the probe-immobilized surface

The probe antibody-modified surfaces were used to detect the SCC-Ag, and Fig. 2B shows the detection of 1 nM SCC-Ag with the antibody. It was noticed that the current level increased from $4.4E^{-08}$ to $7.36E^{-08}$ after adding SCC-Ag. The doubling of the current confirms the interaction of the antibody and SCC-Ag. As there is a clear current increase after adding the SCC-Ag, SCC-Ag was titrated from a picomolar concentration to a nanomolar concentration (60 pM to 1 nM) to evaluate the limits of detection. As shown in Fig. 3A, after the ethanolamine attachment, concentrations of SCC-Ag were added independently on the antibody-modified MWCNT surfaces from lowest to highest. At the lowest concentration of 60 pM (black curve), there is a small variation in the current from $4.4E^{-08}$ to $4.54E^{-08}$. At a concentration of 120 pM (red line), the increase is a little higher than $5.01E^{-08}$. Next, with increasing concentrations up to 240 pM (blue line), the level of the current increases further to $5.53E^{-08}$, but at a concentration of 500 pM (purple line) there is little difference in the current. Finally, the highest concentration of 1 nM (pink line) shows a large difference in the current, which changes from $4.4E^{-08}$ from $7.36E^{-08}$. Figure 3B shows the current

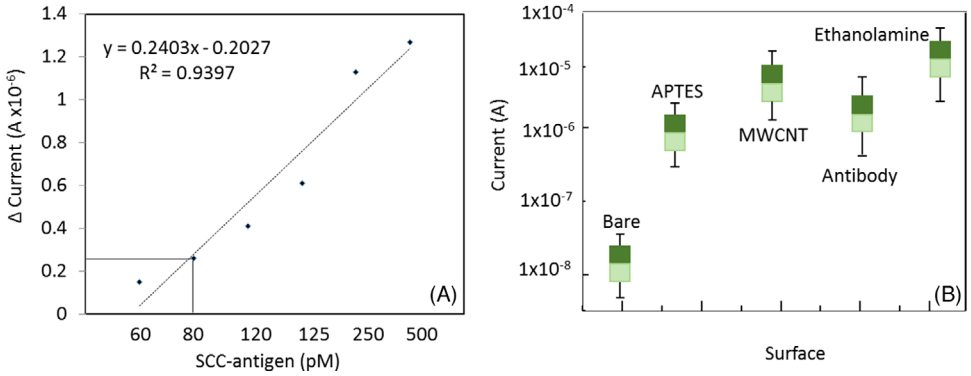

FIG. 4 *High-performance analysis. (A) Linear regression analysis. The limit of detection was determined based on a 3σ estimation. (B) The reproducibility analysis with different biomolecular attachments. The error values are calculated as indicated.*

difference with each concentration of SCC-Ag from the level of the ethanolamine. It was calculated that 60 pM of SCC-Ag shows a difference of 0.14, 125 nM shows 0.61, 250 nM shows 1.13, 500 nM shows 1.27 and 1 nM shows a difference of 2.96. This result shows that there are clear increments when increasing the concentration of SCC-Ag. This result indicates there is an approach to the saturation beyond a concentration of 250 nM. This result clearly defines the limit of detection of SCC-Ag to be in a range between 60 and 120 pM (Fig. 4A). Further, a pin-point titration was performed at 80 and 100 pM, and it was clear that sensitivity was attained at 80 pM (Fig. 4A).

3.4. High-performance analysis on MWCNT-modified IDE surface

From Fig. 4A, the limit of detection was calculated from a linear regression and by a 3σ estimation. It was apparent that the limit of detection is ~80 pM, which is a good sensitivity. To demonstrate the high-performance detection, the above tests were repeated, and the error range was estimated. From Fig. 4B, it is obvious that there were no significant differences in all of the above binding events after repetition, which proved the high performance of the sensor with this proposed strategy. Further, the specific detection of SCC-Ag was carried out with two related, controlled proteins: human blood clotting factor IX and HSA. Figure 5 clearly shows the specific SCC-Ag interactions with the anti-SCC-Ag antibody. With other proteins, the anti-SCC-Ag antibody did not show any significant interactions with current changes. This result confirms that the method of detection does not show any biofouling effects on the MWCNT-modified surfaces and only recognizes the SCC-Ag against the anti-SCC-Ag antibody. Further, the stability of the probe was tested for 8 weeks, and no significant reductions were noticed until the 7th week. At the 8th week there was a 10% reduction in the signal.

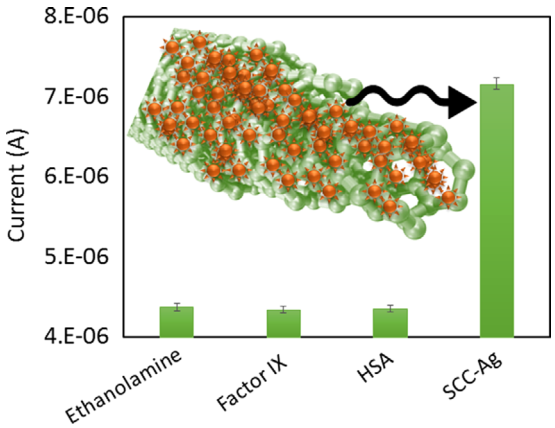

FIG. 5 *Specific interaction of SCC-Ag. The detetion was performed on the MWCNT-modified surfaces. Control experiments were carried out with two different proteins (Factor IX and HAS). SCC-Ag only interacted with its antibody and increased the current level.*

4. Conclusions

Ovarian cancer is a fatal gynecological cancer. It starts in the ovaries and then moves into other parts of the reproductive system. SCC-Ag has been found to be a suitable biomarker to identify ovarian cancer. Carbon-based nanomaterials have also been proven to help improve the detection of this biosensor due to their beneficial thermal, physical, and electrical properties. In this work, a MWCNT-modified dielectric interdigitated electrode sensing surface was used to detect SCC-Ag, and the limit of detection of SCC-Ag was found to be 80 pM. Moreover, the specificity experiments with control proteins showed evidence that the detection of SCC-Ag on the MWCNT-modified dielectric surface occurs without biofouling. This dielectrode surface can be used for other potential medical biomarkers in high-performance analyses.

5. Acknowledgements

The funding was provided by the National Natural Science Foundation of China (No. 81000631) and the Natural Science Foundation of Shandong Province (No. ZR2011HQ045).

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