



Voltammetric determination of human papillomavirus 16 DNA by using interdigitated electrodes modified with titanium dioxide nanoparticles

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Received: 13 December 2018 / Accepted: 14 April 2019 / Published online: 8 May 2019
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Abstract

A gene sensor for rapid detection of the Human *Papillomavirus* 16 (HPV 16) which is associated with the appearance of cervical cancer was developed. The assay is based on voltammetric determination of HPV 16 DNA by using interdigitated electrodes modified with titanium dioxide nanoparticles. Titanium dioxide nanoparticles (NPs) were used to modify a semiconductor-based interdigitated electrode (IDE). The surface of the NPs was then functionalized with a commercial 24-mer oligomer DNA probe for HPV 16 that was modified at the 5' end with a carboxyl group. If the probe interacts with the HPV 16 ssDNA, the current, best measured at a working voltage of 1.0 V, increases. The gene sensor has a ~0.1 fM limit of detection which is comparable to other sensors. The dielectric voltammetry analysis was carried out from 0 V to 1 V. The electrochemical sensitivity of the IDE is $2.5 \times 10^{-5} \mu\text{A} \cdot \mu\text{M}^{-1} \cdot \text{cm}^{-2}$.

Keywords Voltammetric assay · Human *Papillomavirus* · Interdigitated electrodes · DNA · Titanium dioxide

Introduction

Cervical cancer is the second biggest cause of malignancy cancer-related death in women around the world, and it happens following the constant infection, with a particular subset of Human *Papillomavirus* (HPV) type [1–3]. HPV contains double-stranded DNA as a genetic material that

belongs to the family *Papillomaviridae*. HPV strains contain more than 100 types, including high and low-risk types, have been identified so far. Fifteen strains of HPV have been identified to keep women at high risk for cervical cancer [4]. Cervical cancer infection is stimulated by HPV high-risk type strains 16, 18, 31, 33 and 35 [5, 6]. HPV 16 is the worst strain for the progression of cervical cancer followed by 18, 31, 33 and 35. Cervical cancer infections start when the HPV virus is in the cervix then entered cells through microabrasions and infect the cells. Several weeks after infection, the virus replicates and spread through the cells. Cervical cancer can be treated if it can be detected at an early stage. HPV associated cervical cancer has symptoms such as vaginal and contact bleeding and vaginal mass may show the existence of malignancy [7].

Serological methods were used for recognition of HPV IgG and IgM antibodies. However, sufficient production of antibodies needs 5 days after the existence of sickness symptoms. Besides that, serological test suffered from cross contamination reaction with other HPV strains due to antigenic determinants on polyclonal antibodies. Supplementary Table S1 showed comparison of the performance of detection of HPV and cervical cancer by using various methods.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00604-019-3445-2>) contains supplementary material, which is available to authorized users.

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Characterization of disease based on their unique hereditary genetic profiles is expanding and being studied as compared to more conventional strategies of disease characterization. Currently, virological techniques combined Polymerase Chain Reaction (PCR) for the amplification of specific genes are chosen as they are sensitive, low-risk cross-contamination and less assay time compared to serological procedures [8]. However, the identification methods including stained agarose gel electrophoresis for visualization of PCR product. With arising of nanotechnology today, the advance of nanobiosensor is replacing such traditional methodology. Nanobiosensor based on nucleic acid hybridization has been produced for economical and fast detection of DNA.

Nanoparticles and electrode materials which are biocompatible and had good conductivity have been chosen for biosensor studies [9–14]. The nanobiosensor as electronic systems for cancer detecting has to gain wide enormous application in biomedical and biosensor applications. Electrode materials which are biocompatible and had good conductivity have been chosen for biosensor studies [11–14]. The detection of HPV by using TiO₂ as a semiconductor in IDE biosensor was required by consuming low fluid volume, high response, better process control, high surface-volume ratio, reusable, compact system and fast diagnose steps. In the present study, microfabricated TiO₂ based interdigitated electrode (IDE) sensor was utilized as a solution for all these constraints that have been faced by the currently available techniques [15]. This electrical based nanobiosensor integrated with biomolecule probes allowed to detect the changes in charge when HPV DNA target binds to the immobilized probe. TiO₂ based IDE proved to be sensitive, direct and will be suitable for the specific detection of a large species are suffering from cancer. It has a quick response time, low detection limitation and label-free method, make this device to be different from previous detections. This study explores the attainability of HPV DNA detection on TiO₂ based IDE nanobiosensor offering a low fluid volume consumption, high-throughput analysis and response, fast detection, better process control, high surface to volume ratio, sensitive and selective, reusable, compact system, cost-effective and fast diagnosis.

Materials and methods

Chemicals and reagents

3-Aminopropyltriethoxysilane (APTES) was bought from Sigma–Aldrich, USA. It was stored in a dry, ventilated place and firmly sealed before use. Single-stranded carboxylated 24mer (Genbank Accession No.: A18875.1) oligonucleotide probe composed from Human Papillomavirus (HPV) for the recognition of target DNA HPV 16 was synthesized commercially [16]. Table 1 shows the list of sequences for 24-mer

probe, complementary, and non-complementary target oligonucleotides. The 24mer probe was utilized as a model framework for immobilization, while other 24mer strands were utilized for target analysis. The supplied lyophilized DNA samples were diluted in deionized distilled water to reconstitute before use.

Interdigitated electrode (IDE) fabrication

IDE nanosensor was fabricated according to Nadzirah et al. [15]. The IDE fabrication process involved nanobiosensor was explained details in Supplementary Method S1: Interdigitated Electrode (IDE) Fabrication. Supplementary Fig. S1 showed the schematic diagram of the fabrication process involved eight steps. Schematic diagram of recognition of biomolecules on synthetically treated silicon substrates utilizing APTES is shown in Supplementary Fig. S2. The strategy includes: (a) silicon substrates cleaning by piranha solution; (b) silane layer surface modification using APTES (24%, v/v) solution; (c) titanium dioxide (TiO₂) deposition [15]; (d) HPV DNA Probe immobilization; and (e) HPV DNA target hybridization. Preparation of TiO₂ solution and deposition was followed according to Nadzirah et al. [15]. Very thin aluminum metal was deposited on the pads area so that it can cover that area from TiO₂ film deposition [15, 18]. Deposition of TiO₂ methods was explained detail in Supplementary Method S2.

Surface modification on TiO₂ based IDE

TiO₂ based IDE was functionalized using APTES as a linker and also facilitator to immobilize biomolecules on the inorganic surface. 1.5 μl APTES (24% in deionized distilled water) was dropped on an active site of TiO₂ based IDE device. TiO₂ based IDE device was drying in a dry cabinet for 15 min until it completely dry. After that, TiO₂ based IDE device was ready for electrical measurement.

Immobilization of human papillomavirus (HPV) DNA probe

A modified of the 5' end probe with a carboxyl group (COOH) was needed onto the surface since APTES has amine terminated functional group toward the end. HPV Probe DNA (1 μM) which was diluted in deionized distilled water was dropped in the active area. The device was incubated for 2 h in a moist environment at an ambient temperature. Excess DNA probe was rinsed free from the surface by washing the device 3 times with the intervals of 5 min using deionized distilled water. After the immobilization process, TiO₂ based IDE was ready for electrical measurement.

Table 1 DNA sequences for HPV DNA probe oligonucleotide and complementary 24-mer analyte HPV16A-CMP (AIT Biotech, Singapore) [10, 16, 17]

Name	Sequences
HPV16A oligonucleotide probe	COOH 5'-GGG GTC GGT GGA CCG GTC GAT GTA-3'
HPV16A complementary target	5'-TAC ATC GAC CGG TCC ACC GAC CCC-3'
HPV16A non-complementary	5'-ATG TAG CTG GCC AGG TGG CTG GGG-3'
HPV16A single mismatch	5'-TAC ATC GAC CGG TGC ACC GAC CCC-3'

Hybridization of HPV 16 DNA target

The DNA of HPV 16 was initially denatured into by heating in thermoshaker at 95 °C for 5 min after that stop the reaction by keeping on ice for 5 min. One microliter of this single-stranded DNA was then dropped on the active area of IDE and incubated for 1 h. Unbound DNA was removed from the surface by washing the chip.

Testing different concentrations of HPV DNA target

In this study, different concentrations of HPV DNA target in the buffer were prepared to test the detection limit of TiO₂ based IDE. Different concentrations of target DNA (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶ and 10⁻⁷ μM) were prepared from 1 μM HPV DNA target stock solution, by serial dilution with an appropriate volume of deionized distilled water. 1 μl sample was dropped on the active area of IDE by using micropipette. After complementation and washing, the IDE device was ready for electrical measurement.

Electrical measurement

Specifically, after the immobilization of the HPV 16 DNA probe, TiO₂ based IDE current was measured by probing two terminal probes. TiO₂ based IDE substrate device was being able to be monitored through the current flowing to the channel. It also flew to the substrate when the source electrode voltage was swept. A single range of voltage was supplied to the source of the terminal in interims of 0.05 V with a consistent set at 100 mA. At the same time, the current was measured and showed as a current-voltage (I-V graph). Hybridization measurements are taken using current-voltage (I-V) (KEITHLEY, 6487) to determine the change in current flow for TiO₂ based IDE active area before and after DNA hybridization.

High-performance analytical tests on TiO₂ based IDE sensing surface

To test the reproducibility on the TiO₂ based IDE sensor surfaces, 4 different devices of nanogaps were modified with APTES and then HPV DNA probe was immobilized. On the surfaces, 1 μM of complementary HPV DNA target was hybridized and measured in the electric flow. The repeatability

test was done for four TiO₂ based IDE for four different concentrations of single-stranded HPV DNA target (10 μM, 10 nM, 10 pM, 10 fM).

Validation assay using signal amplification

Validation assay using signal amplification was referred according to HC2 High-Risk HPV DNA Test® [19]. There were 8 patient clinical specimens had been tested to validate the effective of fabricated IDE biosensor. Digene Microplate Luminometer 2000 (DML 2000™) Instrument was switched on at least 60 mins before the signal detection. The DR2 (75 μl) was pipetted into a new falcon tube by using multi-channel pipette with reverse pipetting technique into each wells of micro plate. The micro plate was incubated for 15 min in dark places to avoid direct sunlight for 15 mins. The microplate was read on the Digene Microplate Luminometer 2000 in 15 min and not exceeds 30 min.

Results and discussion

Titanium dioxide (TiO₂) was the commonly researched single-crystalline framework in the surface science investigation of metal oxides [20–23]. Semiconductor TiO₂ has adequately positive valence band edge to oxidize water to oxygen [24]. It is also an addition stable material in the presence of aqueous electrolyte solutions. TiO₂ is moderately cheap, exceedingly stable chemically, and the photo-generated holes are very oxidizing. TiO₂ which was inorganic nanoparticles has a decent potential to be utilized as a base component and a part of the biosensor to distinguish the occurrence of biomolecules. This may permit TiO₂ nanoparticles to be utilized as a part of our innovation of biomedical and clinical applications [25, 26]. To make a genuine application conceivable with clinical examples, the target DNA sequence would be longer as genome sequences after being restricted by restriction enzyme [27]. HPV contains virion that has a double-stranded, circular DNA genome of around 7900 bp, and is separated into 3 regions, region of the non-coding long control (LCR, ~1 kb), and the regions of protein-coding early (E, ~4 kb) and late (L, ~3 kb). The viral genome encodes 6 early (E1, E2, E4, E5, E6, and E7) and two late (L1 and L2) proteins. By synthesizing appropriate sequence from this genome this

study demonstrated the detection of Human *Papillomavirus* (HPV) 16, as it associated with cervical cancer cells.

TiO₂ based IDE sensor

Initially, the wafer was cleaned and covered with the insulating material. In this research, we utilized SiO₂ as the insulator because it is cheap, broadly utilized, and a good barrier layer for moisture and mobile ions. The band gap (G) between the terminal electrodes was the most important geometric parameter in deciding the distribution and quality strength of the electric field and the current density compared to the width (W) and height (H) of the IDEs. A flimsy layer (400 nm) of positive photoresist (pPR) was deposited, and the undesirable silicon on the wafer surface was etched to form silicon nanogap with sizes of 1 μm utilizing the chrome mask. They were trimmed to the desired nanoscale sizes utilizing plasma processes. The oxides were developed on the surface of the micro-sized electrodes, which consumes the silicon, and is later etched away by the buffered oxide etcher. The amount of silicon used relies on the aggregate infiltration of the oxide, which is constrained by the development of oxygen through the oxide-silicon interface.

The mask design of IDE was performed using AUTOCAD software, and a single IDE is zoomed-in to identify its full specs. The design of interdigitated electrodes is shown below. Supplementary Fig. S1(a) shows mask design of IDE using AUTOCAD software, and a single IDE is zoom-in to identify its full specs. While Supplementary Fig. S1(b) shows the real chrome mask. The size of electrode have been utilized was 5.5 × 0.25 mm, pad size 2 × 2 mm, the gap between end electrode terminal to the pad is 3 mm and a number of electrodes is 20 with the gap size between two electrodes is 10 nm. IDE is a device which comprises two interlocking comb-shaped metallic coatings in the fashion of a zipper. It's made out of two electrodes with two connection tracks, on a silicon substrate. These IDEs offer a few points of interest, for example, working with a low volume of sample and avoiding from repetitive cleaning of solid electrodes. Sensor surface purities due to cleaning process were important to stabilize the uniformity of 3-aminopropyltriethoxysilane (APTES) as Self Assemble Monolayer (SAM) layers [28]. The interdigitated setup ordinarily improves sensitivity and detection limits. They are appropriate for decentralized assays, to create particular (bio) sensors and other electrochemical studies.

Electrical detection of HPV 16 DNA using TiO₂ based IDE sensor

TiO₂ based IDE was functionalized with APTES and 24 mer HPV 16 DNA probe (Table 1). A fragment of HPV 16 identified in the E6 region was selected as the target, which is complementary to the immobilized HPV 16 DNA probe.

After hybridization, HPV 16 DNA target was bound to the DNA probe functionalized TiO₂ based IDE, achieving negative charges to the sensor surface because of resistance change in TiO₂ based IDE. The fundamental detecting mechanism for this study included the current change prior to and after the hybridization of DNA target. DNA conveys a net negative charge when hybridization happens, there would be a change in negative charge on the surface of TiO₂ based IDE. The reaction change prior to and after the hybridization corresponds to the attachment of the HPV DNA target on the TiO₂ based IDE surface. I-V curve was obtained in the measuring solution after HPV DNA probe attachment onto the TiO₂ based IDE sensor surface. The measurement was directed again in the same condition after HPV DNA target was hybridized to the immobilized HPV DNA probe. Figure 1a illustrates the TiO₂ based IDE working I-V curve before and after 1 μM concentration of HPV 16 DNA synthetic target was hybridized to the complementary HPV 16 DNA functionalized TiO₂ based IDE. The TiO₂ based IDE current was dropped, thus the resistance of TiO₂ based IDE expanded after the negative charges were acquainted with TiO₂ based IDE surface because of DNA/DNA hybridization.

Electrochemical impedance spectroscopy (EIS) was added besides voltammetry determination in Fig. 1b to demonstrate the successfully of assemble the biosensor interface. Figure 1b showed the impedance of bare IDE, TiO₂ based IDE, TiO₂ based IDE/APTES, TiO₂ based IDE/APTES/HPV 16 DNA probe (Immobilization), and TiO₂ based IDE/APTES/HPV 16 DNA probe/ HPV 16 DNA target (Hybridization). Impedance measurements can provide valuable information about the kinetics of monolayer formation, and a rough estimate of the dielectric constant. Comparing to voltammetric capacitive biosensors, biosensors based on EIS have been widely explored for their ability to capture complex resistance changes due to binding events at biosensor's electrode sites [29].

To further confirm the current decrease was brought by the hybridization of complementary HPV 16 DNA target to the immobilized DNA probe, 1 μM scrambled DNA sequences were hybridized to the DNA-functionalized TiO₂ based IDE surface. As shown in Fig. 2, TiO₂ based IDE working I-V curves prior to and after hybridization demonstrated the hard change in current, showing no HPV 16 DNA scrambled DNA sequences were hybridized to the TiO₂ based IDE surface.

The synthetic DNA had a lower infectivity than natural DNA, indicating approximately one lethal error per 500 bp. Most of the experiments were performed at pH 7.4 which was near to neutral regardless of pH which has an adverse effect for DNA adsorption. The lack of a good understanding of the sensing mechanism hampers the further exploitation of these promising nano sensors.

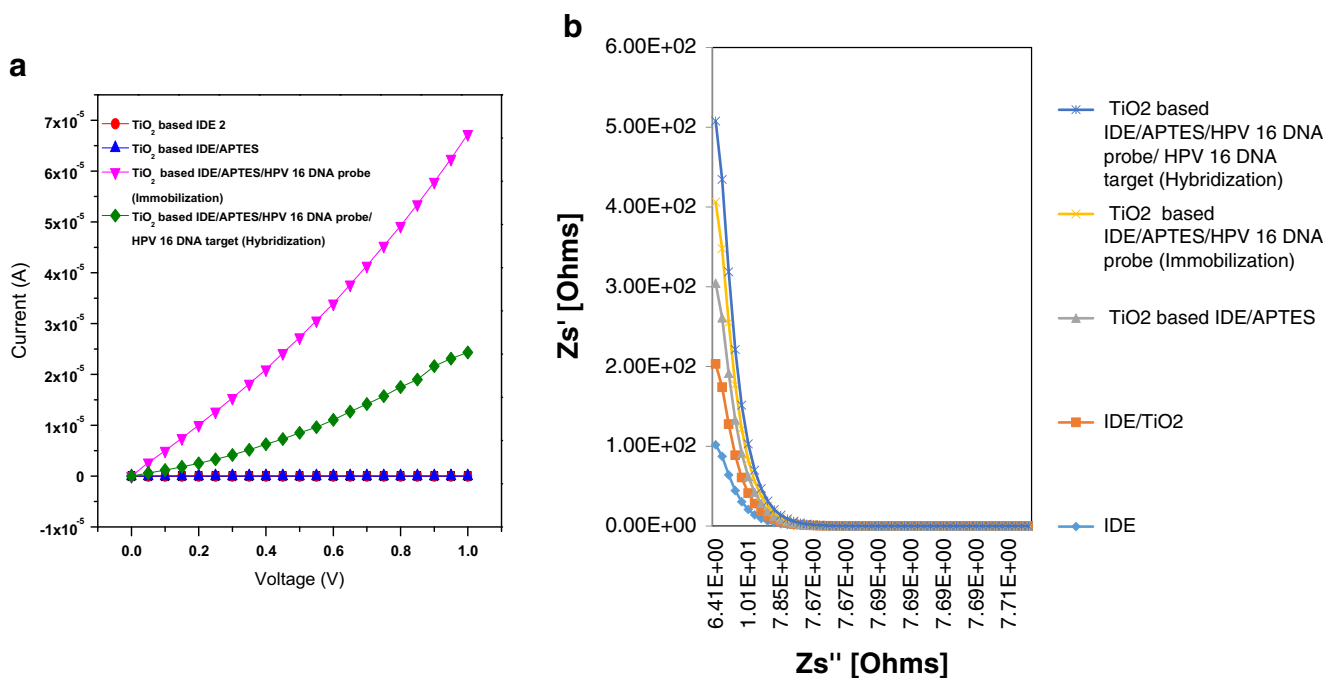


Fig. 1 a Typical TiO_2 based IDE working I-V curves before and after 1 μM of the HPV 16 DNA synthetic target were hybridized to the complementary HPV DNA probe functionalized TiO_2 based IDE surface. b The impedance of bare IDE, TiO_2 based IDE 2, TiO_2 based

IDE/APTES, TiO_2 based IDE/APTES/HPV 16 DNA probe (Immobilization), and TiO_2 based IDE/APTES/HPV 16 DNA probe/HPV 16 DNA target (Hybridization)

Hybridization of HPV DNA target at various concentrations

I-V curves of hybridization of HPV DNA target at various concentrations were shown in Fig. 3. The sensor was tested with the initial trial with a low concentration of HPV DNA and which is able to detect early detection of the disease effectively. TiO_2 based IDE device can detect HPV-associated cervical cancer at different concentrations in order to validate

the device capabilities for detecting pre-cancer development. Using this method, HPV 16 DNA target has detected in the 1 to 10^{-7} μM concentration range without apparent effort to upgrade the system for sensitivity. The concentration 1 μM of HPV DNA probe can detect 1×10^{-4} μM HPV target DNA equivalent to 100 pM (Fig. 3). By further change of DNA hybridization effectiveness, e.g. ionic strength of the electrolyte and ssDNA probe get together, the performance and sensitivity were improved.

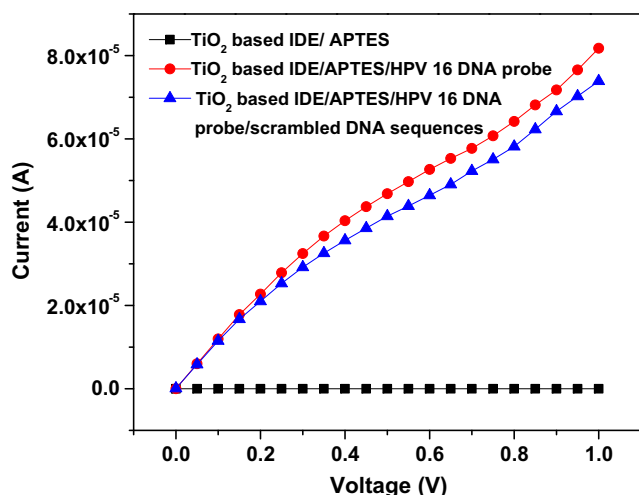


Fig. 2 Typical TiO_2 based IDE working I-V curves before and after 1 μM concentration of scrambled DNA sequences was hybridized to the same HPV 16 DNA functionalized TiO_2 based IDE surface

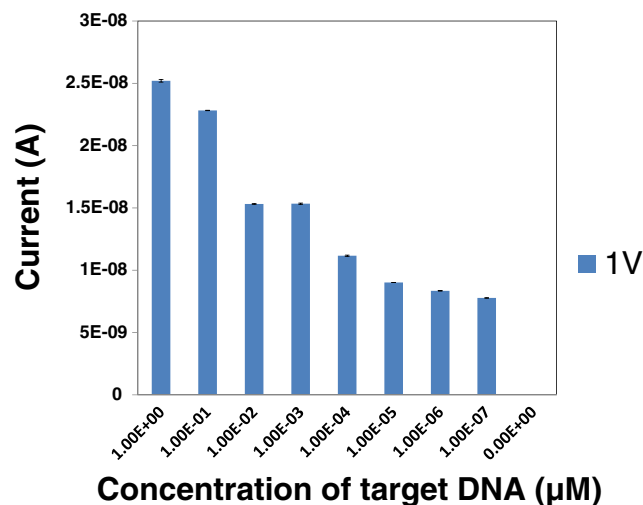


Fig. 3 Serial dilution of HPV DNA target (μM) against current (nA) at 1 V. Error bars represent standard deviation of triplicate determinations of three independent repeated experiments ($n = 3$)

Analytical performance of TiO₂ based IDE

We further tested the analytical performance of TiO₂ based IDE, as shown in Fig. 4. Figure 4a showed the result of I-V characteristic s showed significant differences in the measured current (A) values for each of DNA hybridization. It was observed, that upon hybridization with a complementary target, a decrease in current was recorded. There was no change in the current (A) for non-complementary and single mismatch compared with the current of immobilized DNA probe at 1 V. The decrease in current (A) was associated with DNA hybridization and indicated that DNA is negatively charged because they have phosphate backbone. The use of a DNA probe due to phosphate backbone, it can be immobilized with

p-type semiconductor based transistor surface. Hybridization of a DNA strand from solution was converted to a current response within the Debye length and depends on the analyte concentration, composition, and pH [49]. Stronger binding between DNA duplex due to hydrogen bonding resulted in a higher number of negatively charged DNA bound to surface, so a larger current change happened in this study.

To further verify the device specificity, the relative change in conductance of the device was plotted (Fig. 4b). The change in conductance was observed when 10 μM of the non-complementary target was applied to immobilized DNA probes. The sensitivity and Limit of Detection (LOD) of the TiO₂ based IDE were shown in Fig. 4c and d. The effect of different concentration of complementary target HPV DNA,

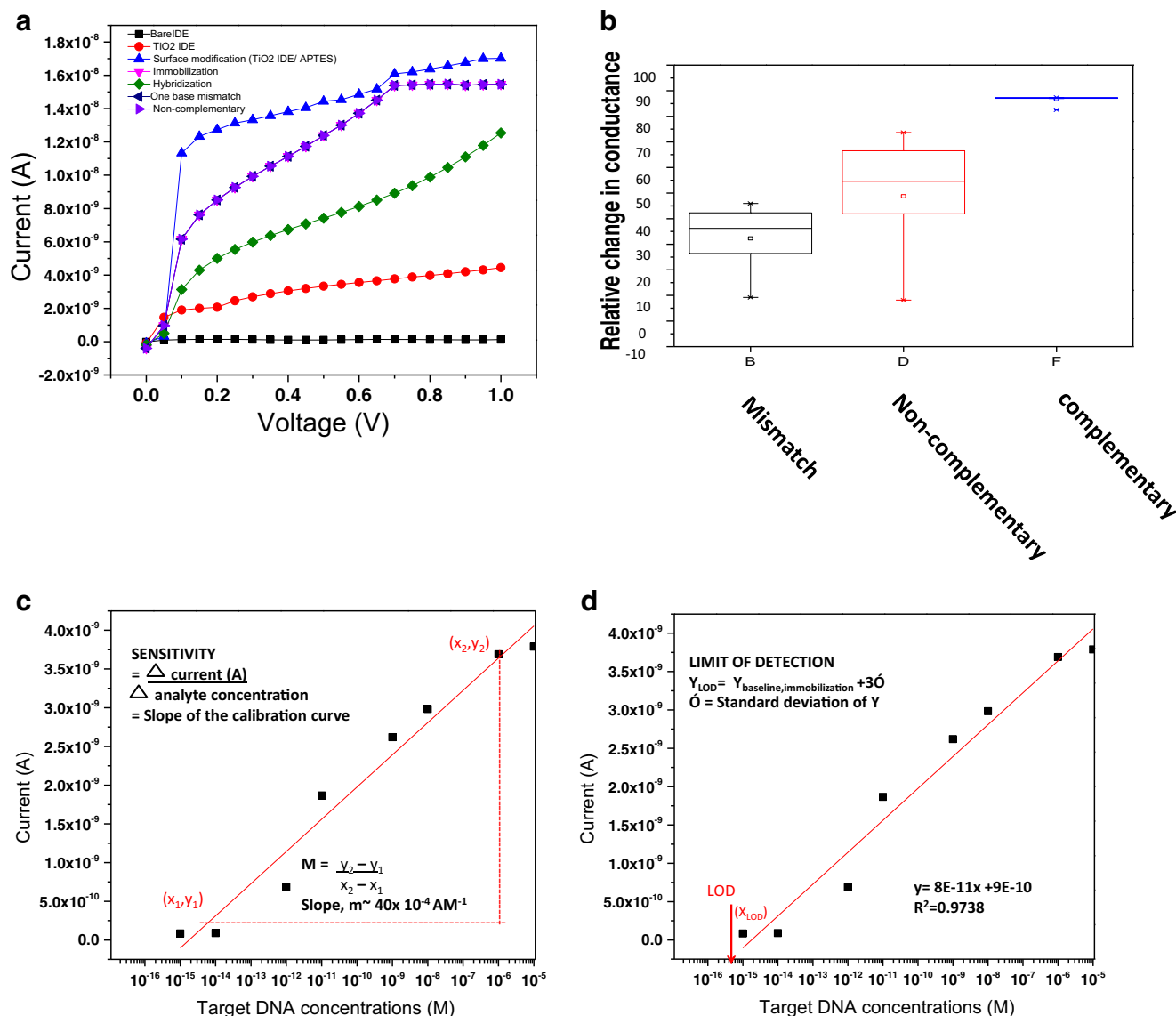


Fig. 4 TiO₂ based IDE biosensor; **a** I-V characteristics by different steps of surface functionalization. **b** Hybridization specificity demonstrated by the conductance to the complementary, one-base mismatched, non-complementary DNA sequences at 1 V. **c** Current response curve of

TiO₂ based IDE biosensor with different concentrations of HPV DNA. **d** Calibration graph of the relative change in current, display limit of detection (LOD)

ranging from 10 fM to 10 μ M was studied. The I-V characteristics in Fig. 4c indicated the current increased with increment of target HPV DNA concentration. By adding more negative charge on the surface, p-type of TiO₂ based IDE accumulated of charge carrier around TiO₂ based IDE. The sensitivity of TiO₂ based IDE device was the slope of the calibration plot at Fig. 4c. The electrochemical sensitivity of

$2.5 \times 10^{-5} \mu\text{A} \cdot \mu\text{M}^{-1} \cdot \text{cm}^{-2}$ and it became saturated after that. LOD can be used to evaluate the ability of the device to detect and predict the lowest concentration of an analyte in the clinical target of HPV DNA. A calibration graph was plotted in Fig. 4d showed the relative change in current proportional to the HPV DNA concentration. To the best of our knowledge, the sensitivity and LOD required are among the best in the detection of HPV DNA compared to previously reported as shown in Supplementary Table S1.

Analytical performance of repeatability, reproducibility, and stability of the biosensor

The capability of the TiO₂ based IDE for repeated HPV DNA detection which was considered as repeatability performance was investigated. The sensor was treated with 0.01 M NaOH for 2 min to dehybridize DNA duplex on the TiO₂ based IDE, which was back to the condition with the probe. The repeatability test was done on the different TiO₂ based IDE device as shown in Fig. 5. The current values for 5 cycles showed no significant changes with slightly differences in hybridization and dehybridization. Relative standard deviation (R.S.D.) of hybridized and dehybridized cycles for regeneration cycles was found less than 5% for the same TiO₂ based IDE. Good repeatability of the TiO₂ based IDE sensor was shown from this result. A further test was examined to analyze the reproducibility of the sensor by comparing 5 different TiO₂

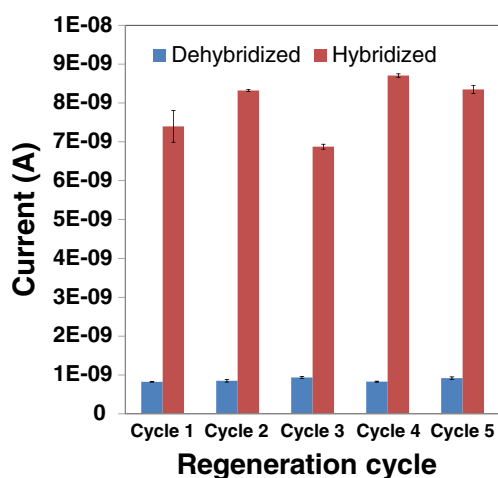


Fig. 5 Repetitive cycles on TiO₂ based IDE biosensor using 10 μ M target HPV DNA

based IDEs as in Supplementary Fig. S2. The results showed a satisfactory reproducibility performance of the sensor with R.S.D. larger at 25% compared to repeatability performance. This result due to minor variation in the TiO₂ based IDE dimension and also the purity of the nanomaterial that being used in the experimental procedures. The TiO₂ based IDE has promising values in the monitoring of HPV DNA with stable and excellent repeatability and reproducibility for stability performances of biosensors.

Validation experiments were carried out using commercializes methods, Hybrid Capture II (HCII) to confirm the effective of fabricated IDE biosensor. Figure 6 showed analytical performance of the hybrid capture assay for HPV. E6 dependence of chemiluminescent signal output for 8 cervical scrapes specimen. Specimen 2 was identified as positive while analytical performance of chemiluminescent signal output zoomed out for negative cervical scrapes specimen.

Conclusion

As a proof of concept in the study, TiO₂ based IDE nanobiosensor utilizing nucleic acid hybridization and electrical detection has been produced with specific and sensitive detection of HPV 16. It is shown that this electrical biosensor was able to detect as low as 0.1 fM concentration (LOD) with a greatly enhanced sensitivity of $40 \times 10^{-4} \text{AM}^{-1}$ with high specificity, repeatability, and reproducibility. Compared to the traditional lab-based methodologies including serological and virological test, this assay has a few points of interest. To begin with, TiO₂ based IDE sensor is sensitive compared to another technique. Second, the TiO₂ based IDE sensor permits quick detection than conventional techniques. Third, TiO₂ based IDE is label-free, dispensing additional procedures for labeling. Fourth, TiO₂ based IDE sensor can be made as portable detection by coordinating the sensor with electrical circuits that measure I-V for the development for HPV-associated cervical cancer early detection.

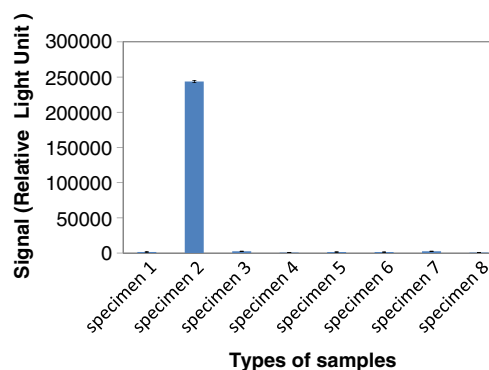


Fig. 6 Analytical performance of the hybrid capture assay for HPV by using real samples

Acknowledgements This research was supported by the Ministry of Higher Education (MOHE) under Grant Nos. FRGS/2/2014/TK03/UNIMAP/01/1. Author would like to thank all staff members of the Institute of Nanoelectronic Engineering in Universiti Malaysia Perlis for their technical advice.

Compliance with ethical standards The author(s) declare that they have no competing interests.

Ethical approval All samples were obtained with the permission from Universiti Malaysia Perlis (UniMAP) Health Centre and acquired with consent from patients who were informed about the research purposes, and this consent procedure was approved by the Medical Ethics Committee for the University Health Centre, UniMAP. The above-mentioned ethics committee specifically approved this study, which was conducted in accordance with the International Conference on Harmonisation–Good Clinical Practice (ICH–GCP) guidelines and the Declaration of Helsinki.

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