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PII: S0003-2670(16)31422-2

DOI: 10.1016/j.aca.2016.11.071

Reference: ACA 234943

To appear in: *Analytica Chimica Acta*

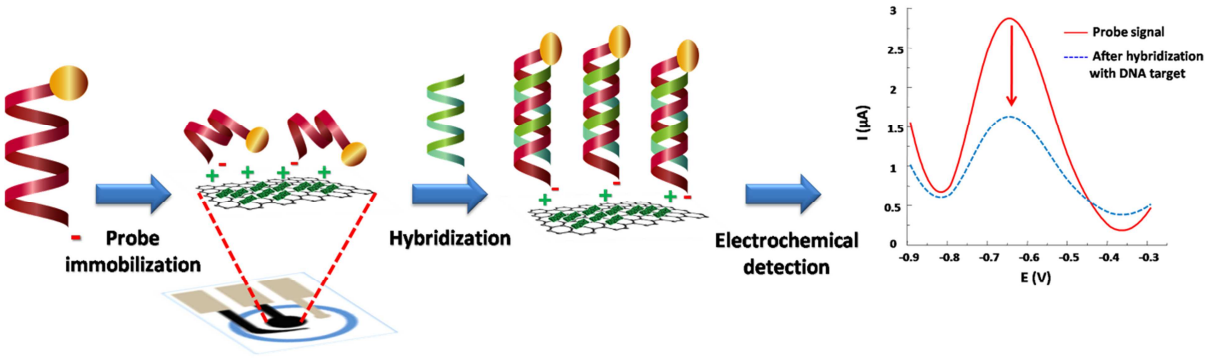
Received Date: 23 July 2016

Revised Date: 21 October 2016

Accepted Date: 29 November 2016

Please cite this article as: P. Teengam, W. Siangproh, A. Tuantranont, C.S. Henry, T. Vilaivan, O. Chailapakul, Electrochemical paper-based peptide nucleic acid biosensor for detecting human papillomavirus, *Analytica Chimica Acta* (2017), doi: 10.1016/j.aca.2016.11.071.

Graphical abstract



Electrochemical paper-based peptide nucleic acid biosensor for detecting human papillomavirus

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ABSTRACT

A novel paper-based electrochemical biosensor was developed using an anthraquinone-labeled pyrrolidinyl peptide nucleic acid (acpcPNA) probe (AQ-PNA) and graphene-polyaniline (G-PANI) modified electrode to detect human papillomavirus (HPV). An inkjet printing technique was employed to prepare the paper-based G-PANI-modified working electrode. The AQ-PNA probe baring a negatively charged amino acid at the N-terminus was immobilized onto the electrode surface through electrostatic attraction. Electrochemical impedance spectroscopy (EIS) was used to verify the AQ-PNA immobilization. The paper-based electrochemical DNA biosensor was used to detect a synthetic 14-base oligonucleotide target with a sequence corresponding to human papillomavirus (HPV) type 16 DNA by measuring the electrochemical signal response of the AQ label using square-wave voltammetry before and after hybridization. It was determined that the current signal significantly decreased after the addition of target DNA. This phenomenon is explained by the rigidity of PNA-DNA duplexes, which obstructs the accessibility of electron transfer from the AQ label to the electrode surface. Under optimal conditions, the detection limit of HPV type 16 DNA was found to be 2.3 nM with a linear range of 10-200 nM. The performance of this biosensor on real DNA samples was tested with the detection of PCR-amplified DNA samples from the SiHa cell line. The new method employs an inexpensive and disposable device, which easily incinerated after use and is promising for the screening and monitoring of the amount of HPV-DNA type 16 to identify the primary stages of cervical cancer.

Keywords: Graphene, Polyaniline, Paper-based electrochemical DNA biosensor, acpcPNA, Electrochemical detection, Human papillomavirus

1. Introduction

The most important factors for diagnostic devices, especially for developing countries, are low cost, simplicity and speed of results for early screening and monitoring of disease biomarkers. To achieve this goal, paper-based analytical devices (PADs) have been widely used as an alternative device design for point-of-care (POC) applications [1-3]. Two detection modes that have been most frequently used with PADs include colorimetric and electrochemical detections. Since first reported by Dungchai et al. [4], PADs with electrochemical detection (ePADs) have increasingly attracted attention as they offer a combination of simplicity, low power requirements, low limits of detection, and ease of quantitation [5-8]. ePADs are therefore an ideal platform for developing sensitive, selective DNA biosensors for point-of-care applications.

In electrochemical DNA biosensors, many different electrode types have been used, including a gold [9-11], hanging mercury drop (HMDE) [12, 13] and various carbon-based materials [14-17]. Carbon is considered a good electrode material due to its low cost, wide potential range, chemical inertness and low background current. Furthermore, carbon electrodes have a fast response time and can be easily fabricated in different configurations. These features make carbon suitable for use in ePAD DNA biosensors. However, the use of micro-scale electrodes as part of ePAD is a major obstacle due to limited sensitivity. To overcome this problem, graphene (G) has been used as a carbon-based nanomaterial and has achieved significant popularity due to the large specific surface area and unique electrochemical properties of G [18-20]. G-based electrodes exhibited superior performance compared to other carbon-based electrodes in terms of their electro-catalytic activity and electrical conductivity [21, 22]. G has also been used in combination with various types of functional materials to fabricate high-performance electrodes. Among them, polyaniline (PANI) is a useful conducting polymer that has been widely used for electronic, optical and electrochemical applications such as enzyme-based biosensors and DNA assays due to its

excellent environmental stability and unusual doping/dedoping chemistry [23-25]. Moreover, PANI improves the dispersion and reduces the agglomeration of the planar sp^2-carbon of G [23, 26]. PANI also possesses free amino groups, which can act as a handle for the covalent immobilization of suitable detection probes via amide bonds [27, 28]. Finally, doped PANI possesses the positive charge of the amino group, which can immobilize negatively-charged probes via electrostatic interactions. Thus, it is a challenge to evaluate alternative systems for immobilization of various bio-recognition elements via electrostatic interaction.

For most electrochemical DNA biosensors, a probe that is designed to detect a specific sequence of target DNA is first immobilized on the electrode. Then, the electrochemical signal of an electroactive species, which was either covalently attached to the probe or added later as an indicator, is recorded and compared before and after the hybridization with the complementary DNA target [12, 29]. The probe is a key parameter that determines the detection selectivity. While most DNA biosensors employ short oligodeoxynucleotide probes, several alternative probes have been used with great success. Peptide nucleic acid (PNA) [30, 31], a synthetic DNA mimic with a peptide-like backbone of repeating N-(2-aminoethyl)-glycine units replacing the sugar-phosphate in natural DNA or RNA, has attracted increasing interest as the probe for electrochemical DNA biosensors [12, 32-34] due to its sequence-specific binding to DNA or RNA, resistance to nuclease and protease enzymes, and strong binding to the target DNA. Recently, Vilaivan's group [35-37] proposed a new conformationally constrained pyrrolidinyl PNA system (known as acpcPNA) that possesses an α,β-peptide backbone derived from D-proline/2-aminocyclopentanecarboxylic acid. This new acpcPNA demonstrates a stronger binding affinity and higher specificity towards complementary DNA compared to DNA and Nielsen's PNA, and there are several applications of acpcPNA as a probe for DNA biosensors [38-40].

Human papillomavirus or HPV is the common virus that can be passed through any type of sexual contact. There are some high-risk types of HPV including type 16 and 18, which can cause

abnormal changes to the cells of the cervix. These changes can lead to cervical cancer which, is one of the most important health problems for woman. The mortality occurring from cervical cancer has continuously increased especially in developing countries that have limited medical facilities [41].

In our previous work [38], the electrochemical sensor based-on acpcPNA probe for HPV detection was reported. Although the good limit of detection (LOD) was achieved, the use of PVC-based sensor was costly and the procedure relies on covalent method for probe immobilization was complicated and time-consuming. Cost is the most critical challenge for disposable POC electrochemical sensors. In this work, using PAD has potential to address all of challenges of disposable sensor in term of low-cost and simple fabrication. Electrostatic immobilization is also a key step of this work. This method eliminated the complicate steps of covalent process and significantly reduced time. Moreover, inkjet printing used for electrode modification step is advantageous due to it provided electrode reproducibility and high pattern resolution [42]. Inkjet-printing is scalable of mass production, while small amount of modifier is wasted in the modification process.

Here, we aim to develop a novel paper-based electrochemical DNA biosensor using an AQ-labeled acpcPNA probe in combination with a G-PANI modified electrode. In this new DNA biosensor, the acpcPNA probe labeled with anthroquinone (AQ) was first incorporated onto a G-PANI-modified ePAD using electrostatic immobilization. In the absence of complementary DNA, a strong signal was observed for the AQ. Hybridization with the complementary DNA target resulted in a decrease of the electrochemical signal that linearly correlated with the concentration of the target. The application of the biosensor to the sensitive detection of HPV DNA type 16 is demonstrated. The proposed method is applicable to the screening and monitoring of HPV-DNA type 16 in the primary stage of cervical cancer, a disease that leads to the death of women around the world, particularly in developing countries that have limited resources for public healthcare.

2. Materials and methods

2.1. Chemicals and materials

Graphene (G) was purchased from A.C.S (Medford, USA). Polyaniline, camphor-10-sulfonic acid ($C_{10}H_{16}O_4S$), glutamic acid and N-methyl-2-pyrrolidone (NMP) were ordered from Sigma Aldrich (St.Louis, USA). Carbon and silver/silver chloride ink were obtained from Gwent group (Torfaen, United Kingdom). The screen-printed block was made by Chaiyaboon Co. Ltd. (Bangkok, Thailand). The labeling electroactive spices, 4-(anthraquinone-2-oxy)butyric acid**,** was synthesized as described previously [39]. Analytical grade reagents, including NaCl, KH_2PO_4, Na_2HPO_4 and KCl, were purchased from Merck and used without further purification. Synthetic oligonucleotides which correspond to partial sequences of HPV type 16 and other types of high risk HPV (HPV types 18, 31 and 33) used in the specificity test, were obtained from Pacific Science (Bangkok, Thailand). The sequences of the DNA oligonucleotides used are shown in Table 1.

Table 1. List of oligonucleotides

Oligonucleotide	Sequence (5'-3')
Complementary DNA (HPV type 16)	5'-GCTGGAGGTGTATG-3'
Non-complementary DNA 1 (HPV type 18)	5'-GGATGCTGCACCGG-3'
Non-complementary DNA 2 (HPV type 31)	5'-CCAAAAGCCCAAGG-3'
Non-complementary DNA 3 (HPV type 33)	5'-CACATCCACCCGCA-3'

The forward (5'-CACTATTTTGGAGGACTGGA-3') and reverse primer (5'-GCCTTAAATCCTGCTTGTAG-3') used for the PCR cell-line samples were purchase from Pacific Science (Bangkok, Thailand). The cervical cancer cell-lines with (SiHa) HPV types 16 were obtained from the Human Genetics Research Group, Department of Botany, Faculty of Science, Chulalongkorn University.

149 *2.2 Apparatus*

150 The electrochemical measurements using square-wave voltametry (SWV) were performed
151 using a CHI1232A electrochemical analyzer (CH Instruments, Inc., USA). A three electrode system
152 was used and the working electrode was a G-PANI-modified screen-printed carbon electrode (4 mm
153 in diameter). The electrochemical impedance spectroscopy (EIS) was carried out using PGSTAT 30
154 potentiostat (Metrohm Siam Company Ltd., Switzerland) in a solution of 0.1 M PBS with the
155 frequency range from 0.1 to 10^{-5} Hz. The Dimatix^{TM} Materials Printer (DMP-2800, FUJIFILM
156 Dimatix, Inc., Santa Clara, USA) was used for the electrode modification. JEM-2100 transmission
157 electron microscope (Japan Electron Optics Laboratory Co., Ltd, Japan) was used for
158 characterization of G-PANI composites. MALDI-TOF MS spectra was performed on a Microflex
159 MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany).
160
161 *2.3 Fabrication of a paper-based electrochemical DNA sensor*
162 A paper-based electrochemical DNA sensor was fabricated using the wax-printing method
163 [43, 44]. Initially, the patterned paper was designed using Adobe Illustrator and printed onto the
164 filter paper (Whatman No.1) using a wax printer (Xerox Color Qube 8570, Japan). The printed
165 paper was then placed on a hot plate (175 ºC, 50 s) to melt the wax and create a hydrophobic
166 barrier. For electrode fabrication, three electrode systems were prepared using an in-house screen-
167 printing method. First, carbon ink was screened as a working electrode (WE) and counter electrode
168 (CE). Afterward, silver/silver chloride was screened as a reference electrode (RE) and conductive
169 pad. The basic design of the paper-based electrochemical DNA biosensor was shown in Fig. S1.
170
171 *2.4 Inkjet-printing of a G-PANI composite modified paper-based electrochemical DNA biosensor*
172 Prior to electrode modification, a G-PANI composite was prepared using a physical mixing
173 method following procedures extracted from relevant literature [45]. Graphene powder (10 mg) was

dispersed in N-methyl-2-pyrrolidone (NMP) and sonicated for 20 h at room temperature. Subsequently, 20 mg of PANI (emeraldine base) was doped with 25 mg of camphor-10-sulfonic acid (CSA) to generate a positively charged amino group and also dissolved in 10 mL of NMP. For the preparation of the G-PANI conductive ink, the solutions of G and PANI were mixed together and stirred for 1 h. The mixture was centrifuged at 5000 rpm and filtered through a 0.43 µm filter membrane before use. The morphology of G-PANI conductive ink was characterized by transmission electron microscopy (TEM).

For electrode modification, the G-PANI composite solution was used as the ink for inkjet printing. The G-PANI conductive ink was loaded into a cartridge and printed onto the working electrode of the paper-based electrochemical DNA sensor using a Dimatix^{TM} Materials Printer (DMP-2800, FUJIFILM Dimatix, Inc., Santa Clara, USA) at 30 °C, 20 V of applied voltage and 25 µM of drop spacing. Modification of the electrode is shown in Scheme 1A. Six layers of G-PANI composite solution were printed onto the surface area of the working electrode. Next, the modified electrode was heated at 65 °C for 30 min to dry the solvents from the G-PANI conductive ink.

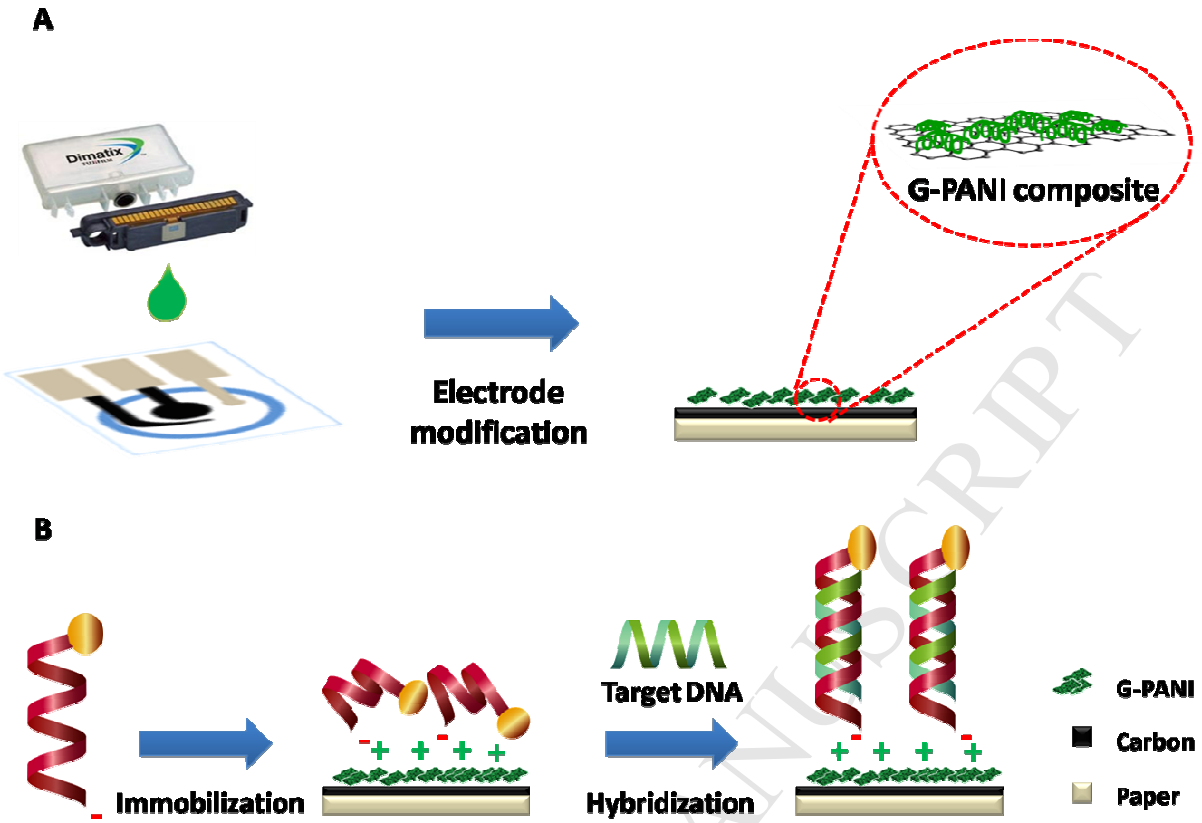
2.5. Synthesis and labeling of the acpcPNA probe

The acpcPNA probe with the sequence Ac-$(Glu)_3$-CATACACCTCCAGC-Lys(AQ)NH_2 (written in the N→C direction, Ac = acetyl; AQ = anthraquinone; Glu = glutamic acid; Lys = lysine) was designed to detect 14 base synthetic oligonucleotide target with a sequence corresponding to human papillomavirus (HPV) type 16. The probe, referred to as AQ-PNA, was synthesized by solid-phase peptide synthesis using Fmoc chemistry as previously described [35]. The probe was modified with three glutamic acid residues at the N-terminus to incorporate the negative charge, which was followed by end-capping with an acetyl group. At the C-terminus, the acpcPNA probe was labeled with anthraquinone (AQ) via 4-(anthraquinone-2-oxy) butyric acid to the amino side chain of a C-terminal lysine residue. The progress of the reaction was monitored by

MALDI-TOF-MS analysis on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The modified PNA on the solid support was treated with 1:1 (v/v) aqueous ammonia:dioxane in a sealed tube at 60 ºC overnight to remove the nucleobase protecting groups. Following cleavage from the solid support with trifluoroacetic acid (TFA), the AQ-PNA was purified by reverse-phase HPLC (C18 column, 0.1% (v/v) TFA in H_2O-MeOH gradient). The identity of AQ-PNA was verified by MALDI-TOF MS analysis, and the purity was confirmed to be >90% by reverse-phase HPLC.

2.6 Immobilization and hybridization of the PNA probe

First, the AQ-PNA probe (3 µL, 125 nM) was immobilized onto the G-PANI modified screen-printed carbon electrode surface by the drop-casting method and incubated for 15 min at room temperature. Next, 50 µL of PBS was placed onto the electrode surface. After immobilization of the probe, the AQ-PNA/G-PANI/SPCE modified electrode was hybridized with 3 µL of target DNA for 15 min. Then, the electrochemical signal response was measured using square-wave voltammetry. The immobilization and hybridization procedures are illustrated in Scheme 1B.



Scheme 1. Schematic illustration of (A) electrode modification and (B) immobilization and hybridization steps of paper-based electrochemical DNA biosensor.

2.7 PCR amplification of the cell line DNA sample

The SiHa (HPV type 16 positive) cell line DNA sample was amplified using PCR as previously reported [38]. Briefly, the amplification mixture containing 0.2 mM deoxynucleotide triphosphate mixture, 1x buffer (KCl, Tris) + 1.5 mM $MgCl_2$, 0.5 U of Taq polymerase, 0.4 µM of each of the primers and 100 ng/µL of the cell line DNA sample was amplified at 95 ºC for 10 min, follow by 30 cycles at 52 ºC for 30 s and finally 72 ºC for 7 min. The success of the PCR was confirmed by gel electrophoresis in 2% (w/v) agarose TBE gel followed by staining with ethidium bromide and visualization under a UV-transilluminator.

2.8 Electrochemical measurement

For electrochemical measurements, the square-wave voltammetry was used throughout the experiment using a potentiostat (CHI1232A). After the immobilization process, 50 µL of PBS was dropped onto the modified working electrode, followed by adding the DNA target. Hybridization with AQ-PNA probe was done for 15 min. The electrochemical detection was performed under the optimal parameters including a frequency of 20 Hz, an amplitude of 100 mV and a step potential of 20 mV. The electrochemical signal after the hybridization was measured and compared with the signal in the absence of the DNA sample.

3. Results and Discussion

3.1 Characterization of the G-PANI conductive ink

To confirm the success of the preparation of the G-PANI conductive ink, the morphology of the ink was characterized by transmission electron microscopy (TEM). Figure S2A shows a TEM image of the G-PANI composite, which indicates a strong dispersion of G inside the composites without aggregation. Moreover, the electron diffraction pattern of G (Fig. S2B) matches very well with the standard, single crystal graphene.

The influence of the G-PANI ratio on the electrochemical conductivity of the modified electrodes was investigated. The anodic current of 1.0 mM $[Fe(CN)_6]^{3-/4-}$ in a 0.1 M KCl solution was measured using different proportions of G and PANI. Interestingly, the anodic peak current of 1.0 mM $[Fe(CN)_6]^{3-/4-}$ increased with an increasing G:PANI ratio. The highest anodic current of $[Fe(CN)_6]^{3-/4-}$ was observed with the G-PANI modified electrode at the ratio of 1:2 indicating the optimal sensitivity of the modified sensor (Fig. S3). In addition, the peak currents gradually decrease for the higher G:PANI ratio, which could be due to the agglomeration of G within the G-PANI modifier [45]. Moreover, the effect of the number of printed G-PANI layers was also

investigated as shown in Fig. S4. The anodic peak current of 1.0 mM $[Fe(CN)_6]^{3-/4-}$ tends to increase with an increase in the number of G-PANI layers. However, the current of the printed G-PANI modified electrode slightly decreased over 6 printing layers. We also believe that this phenomenon arises from the aggregation of excess G on the modified electrode surface causing a decrease in the anodic current response.

Next, the electroactive surface area (A) of SPCE and G-PANI modified electrode were determined to illustrate that the G-PANI conductive ink increases the surface area of the SPCE by solving the Randles-Sevcik equation (equation 1):

$$I_p = (2.69 \times 10^5)\, n^{3/2} A C D^{1/2} v^{1/2} \qquad \text{(equation 1)}$$

Where I_p = current, n = number of electron transfer, C = concentration of electroactive species (mol cm^{-3}), D = diffusion coefficient ($cm^2\ s^{-1}$) and v = scan rate ($v\ s^{-1}$). As shown in Fig. S5, the cyclic voltamograms of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ in a 0.1 M KCl solution for SPCE and G-PANI modified electrode were obtained. The current (i_p) is directly proportional to the square root of scan rate. According to the Randles-Sevcik equation, an approximate value of A was calculated to be 0.041 cm^2 and 0.222 cm^2 for SPCE and G-PANI modified electrode, respectively. This result provided a 5.4 times increase of electrode-electroactive surface area, thus, G-PANI conductive ink can improve the surface area of electrode, leading to enhance the sensitivity. As a result, the six G-PANI-layered modified electrode and the 1:2 ratio of G:PANI were considered to be the optimal conditions and were used for subsequent experiments.

3.2 Characterization of acpcPNA-labeled probe

In order to confirm the success of the synthesis and labeling of the AQ-PNA probe, MALDI-TOF mass spectrometry was used to characterize the unlabeled and labeled acpcPNA probes. The 14-mers unlabeled acpcPNA showed a mass peak at 4754 m/z. After labeling, the mass increased to 5477 m/z. This increase of 723 m/z coincides with the mass of AQ and three glutamic

acid residues plus an acetyl group. Therefore, the labeling of the acpcPNA probe with the electroactive species and the negatively charged polyglutamate was confirmed.

3.3 Electrochemical characterization

Electrochemical impedance spectroscopy (EIS) was used to characterize the AQ-PNA probe immobilization onto the working electrode. In general, the shape of the semicircle portion obtained from the EIS spectrum relates to either the electron transfer limited process or electron transfer resistance. Fig. 1A shows the Nyquist plots obtained from SPCE, G-PANI/SPCE and AQ-PNA/G-PANI/SPCE before and after hybridization with the target DNA in a solution of 1.0 mM $[Fe(CN)_6]^{3-/4-}$. Figure 1A (inset) shows the Nyquist curve of unmodified SPCE (red line) with an R_{ct} value of 27.5 kΩ. This value is larger than that obtained from G-PANI/SPCE, and indicates that the unmodified SPCE has a higher charge-transfer resistance. Therefore, the decrease in R_{ct} to 5.08 kΩ of G-PANI/SPCE (blue line) indicates that G-PANI improves the electron transfer rates. After the immobilization step (Fig. 1A), the semicircular portion of AQ-PNA/G-PANI/SPCE (pink line) dramatically decreased with the R_{ct} value of 2.67 kΩ. This result can be explained theoretically as AQ is electroactive and can improve conductivity. Thus, the AQ-labeled PNA probe, which was immobilized onto the electrode surface, can facilitate the electron transfer process at the interface between the G-PANI/SPCE surface and the electrolyte. For this reason, the Nyquist curve of AQ-PNA/G-PANI/SPCE should have possessed the smallest semicircular portion. In order to confirm the success of the immobilization of the AQ-PNA probe, the heterogeneous electron-transfer rate constant (K_{et}) value was obtained from equation (2), which shows the relationship between R_{ct} and K_{et}.

$$K_{et} = \frac{RT}{n^2F^2R_{et}AC_{redox}} \qquad (2)$$

Where n = number of electron transfer, F = faraday's constant (KJ mol^{-1}), A = electrode surface area (cm^2), C_{redox} = concentration of the redox couple (mol cm^{-3}). From the equation, the K_{et} values for SPCE, G-PANI/SPCE and AQ-PNA/G-PANI/SPCE were calculated to be 7.45 x 10^{-5} cm s^{-1}, 40.28 x 10^{-5} cm s^{-1} and 76.70 x 10^{-5} cm s^{-1}, respectively. The increasing of K_{et} values inferred that the electron transfer process on AQ-PNA/G-PANI/SPCE is easier and faster than that on G-PANI/SPCE and SPCE. These results indicate that the negatively charged AQ-PNA probe was successfully immobilized onto the positively charged G-PANI modified electrode surface through electrostatic attraction.

Next, the electrochemistry of the immobilized AQ-PNA/G-PANI/SPCE probe before and after hybridization with the DNA target were investigated using square-wave voltammetry (SWV), (Fig. 1B). The immobilization of the AQ-PNA/G-PANI/SPCE probe displayed a redox peak at approximately –0.65 V. After hybridization with an equimolar quantity of the complementary target DNA, the electrochemical response significantly decreased due to the increased rigidity of the PNA-DNA duplex relative to the native PNA probe. The rigidity hinders the electron transfer between the redox-active label (AQ) and the electrode surface [46, 47].

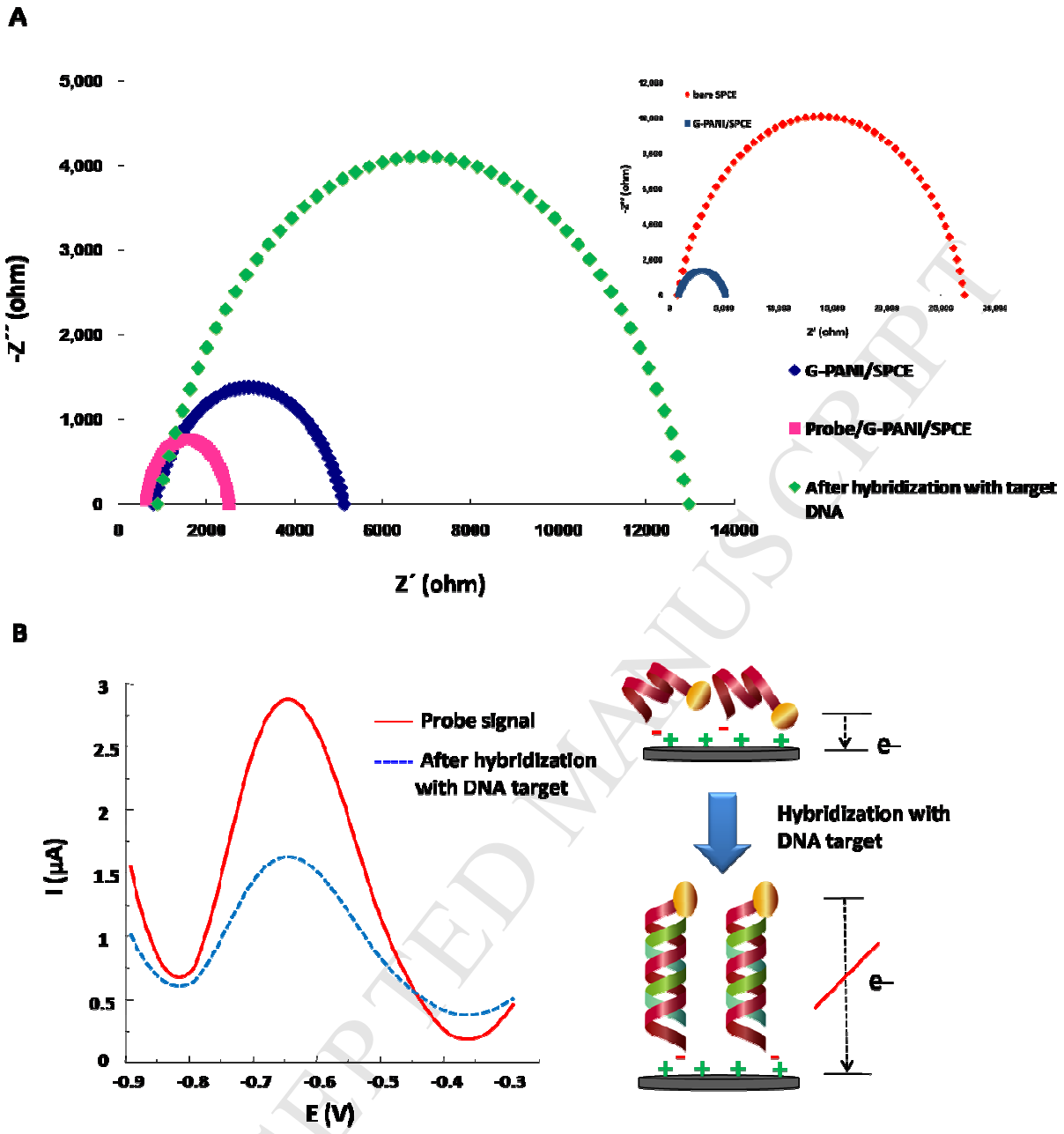
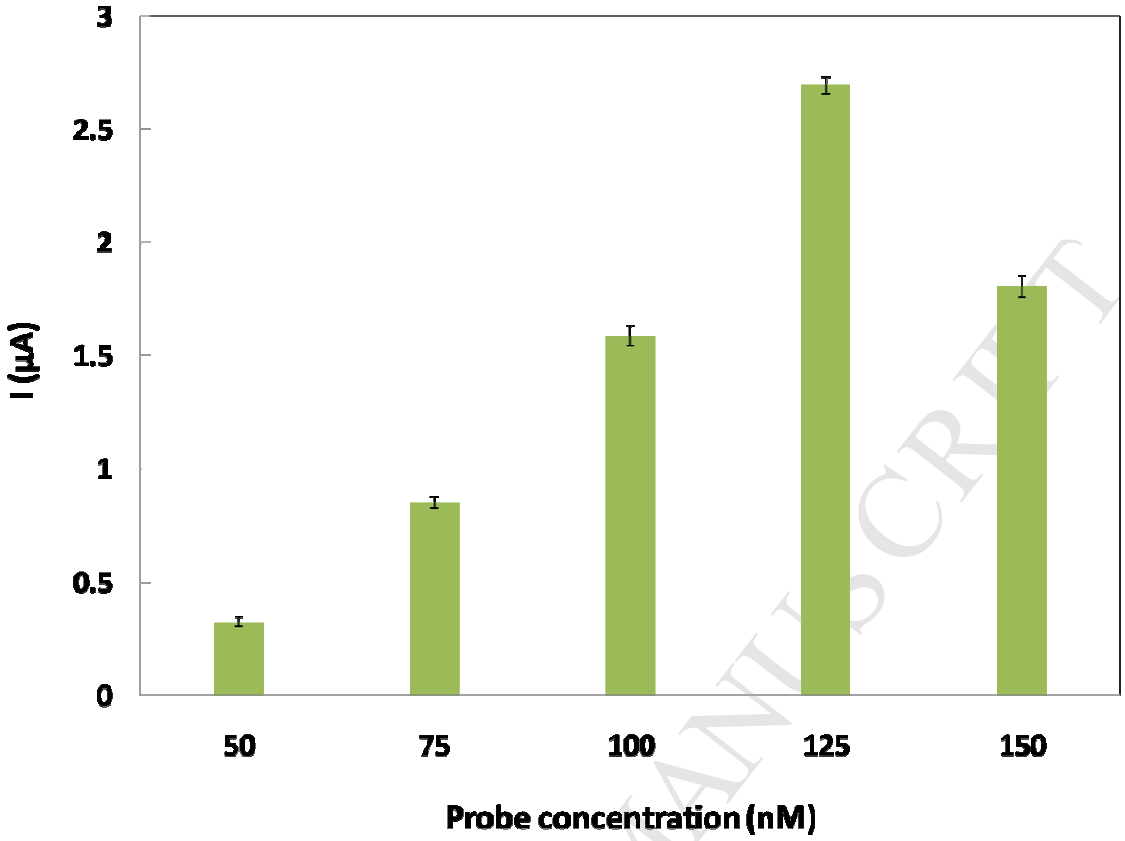


Fig. 1. (A) Nyquist plot of SPCE (inset), G-PANI/SPCE (inset), AQ-PNA/G-PANI/SPCE before and after hybridization with target DNA in 0.1 M $[Fe(CN)_6]^{3-/4-}$. (B) Square-wave voltammograms of immobilized AQ-PNA probe on G-PANI/SPCE before and after hybridization with an equimolar concentration of target DNA.

3.4 Optimization of experimental variables

The experimental conditions were optimized next. The effect of AQ-PNA probe concentration on AQ electrochemical oxidation was investigated first. The oxidation current obtained for different concentrations of the AQ-PNA probe measured by square wave voltammetry are shown in Fig. 2. The current continuously increased up to 125 nM. Above 125 nM, the current decreased significantly. The decrease in peak current at high AQ-PNA concentrations can potentially be caused do to an increased thickness of the organic layer [48, 49], which leads to a lower electron transfer between AQ and the working electrode surface. Hence, the probe concentration of 125 nM was selected as the optimal concentration for further experiments.

For electrochemical detection using SWV, the variable parameters including frequency, step potential and amplitude were optimized using a 125 nM AQ-PNA probe concentration without DNA target shown in Fig. S6. The optimal parameters for electrochemical detection of this system was found to be 20 Hz of frequency, 100 mV of amplitude and 20 mV of step potential.



339

340 **Fig. 2.** The effect of AQ-PNA probe concentration on AQ electrochemical oxidation during the
341 immobilization step.

342

343 *3.5 Analytical performance*

344 To evaluate the analytical performance of the AQ-PNA/G-PANI/SPCE modified electrode,
345 different concentrations of DNA target were determined using SWV analysis. Figure 3 shows the
346 voltammograms as well as the calibration curve (inset) as a function of target DNA concentrations.
347 The calibration curve provided a linear range from 10 nM to 200 nM with a correlation coefficient
348 of 0.997. The limit of detection (LOD) and limit of quantitation (LOQ), which were calculated as
349 the concentration that produced a signal at 3 times and 10 times of the standard deviation of the
350 blank (N=5) [4], were found to be 2.3 nM and 7.7 nM, respectively. Our proposed method provides
351 a wide linear range and sufficiently low detection limit for HPV detection. Table S1 shows a

comparison of electrochemical performance between AQ-PNA/G-PANI/SPCE and the other modified electrodes used for HPV detection. It can be seen that a sufficiently low detection limit for HPV detection could be obtained from our proposed method. Importantly, this ePAD DNA biosensor can be easily and inexpensively prepared compared to the other HPV DNA biosensors [38, 48, 50].

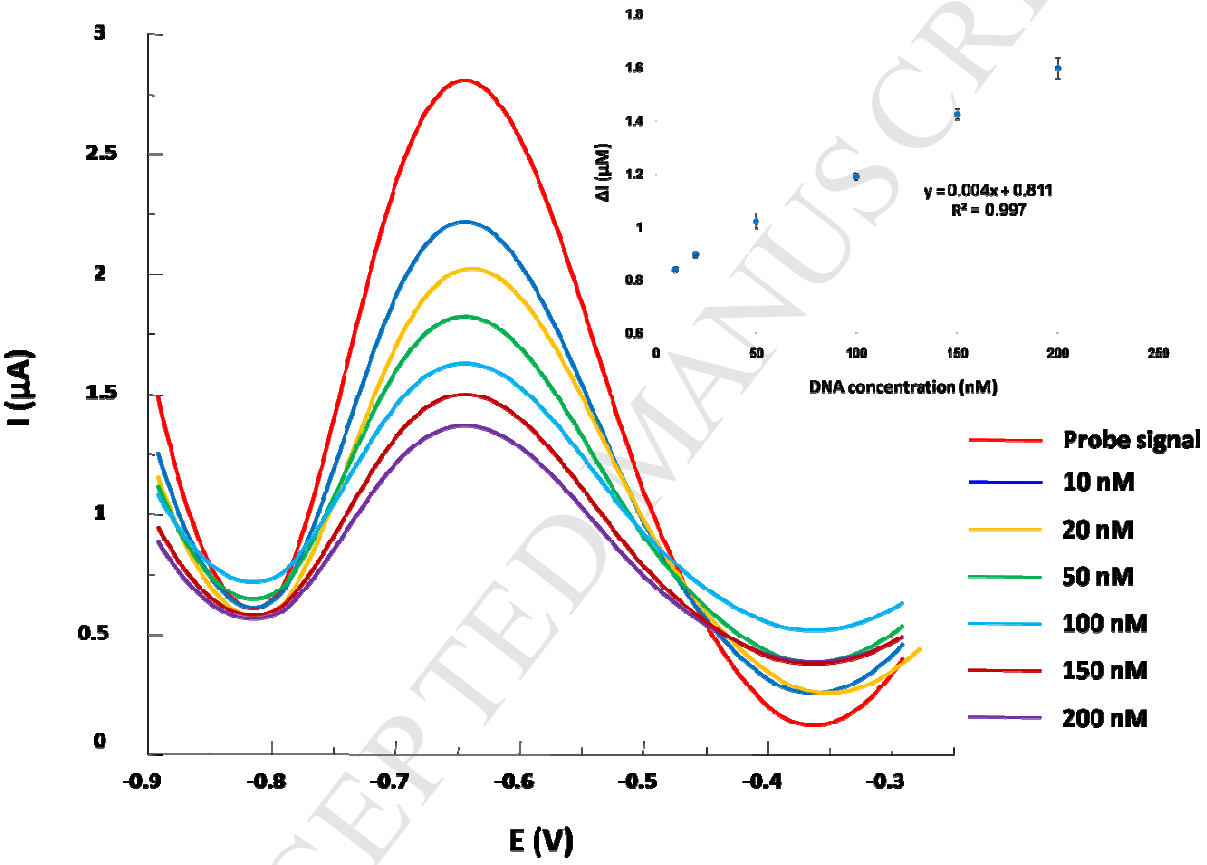
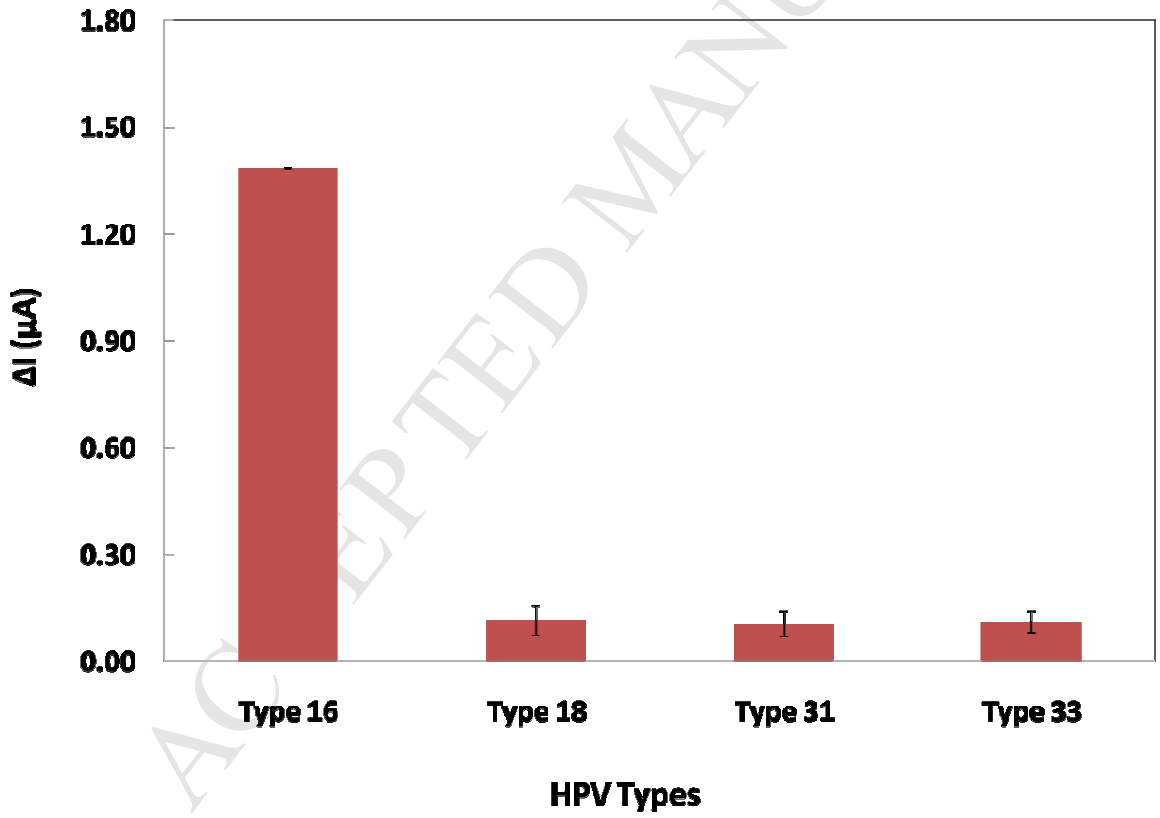


Fig. 3. Square-wave voltamograms of AQ-PNA/G-PANI/SPCE after hybridize with the target DNA in the concentration range of 10-200 nM and the calibration plots of the change in the probe electrochemical current (ΔI) versus the target DNA concentration (inset) at optimal condition; 125 nM of AQ-PNA probe concentration, a frequency of 20 Hz, an amplitude of 100 mV and a step potential of 20 mV.

366 *3.6 Selectivity of the HPV type 16 detection*

367 In order to investigate the selectivity of the acpcPNA probe, the current response obtained
368 from the 14-nucleotide oligomer HPV type 16 DNA target was compared to non-complementary
369 14-nucleotide oligomers, which originate from the other types of high risk HPV (types 18, 31 and
370 33), under the same experimental conditions. As shown in Fig. 4, a significantly different current
371 was only obtained from the complementary DNA relative to the non-complementary DNA.
372 Therefore, the immobilized AQ-PNA probe selectively binds to the HPV type 16 DNA target
373 sequences. Accordingly, the proposed DNA biosensor demonstrated high selectivity to HPV type
374 16 DNA.



375

376 **Fig. 4.** The electrochemical signal response derived from AQ-PNA/G-PANI/SPCE probe after
377 hybridized with various HPV types.

378

3.7 Reproducibility and stability of the paper-based electrochemical DNA biosensor

The electrode-to-electrode reproducibility and stability of the paper-based electrochemical DNA biosensor was examined. The relative standard deviations (RSDs) of five electrodes were tested in the concentration range of 10-200 nM. The % RSDs was determined to be between 2.16% and 7.79%. These results indicate that the proposed DNA biosensor offers acceptable reproducibility. Moreover, the storage stability is another important parameter for DNA biosensor development. The ePAD DNA biosensor was stored at room temperature (25 ºC) for 2 weeks before recording the current response to 10 nM target DNA. It was determined that 90.2% of the initial current response was retained in the aged sensor compared to the response obtained from the freshly prepared sensor. The stability of this DNA sensor is mainly attributed to the environmental stability of PANI.

3.8 Detection of the PCR DNA sample

To test the ePAD biosensor, DNA was extracted from the SiHa cell line, HPV type 16 and amplified using PCR. Figure 5A shows the SWV response of the proposed DNA biosensor in the presence of positive PCR products. It was observed that the current response decreased in the presence of the PCR product from the HPV type 16-positive cell line. Moreover, the current response decreased with increasing amounts of sample, as shown in Fig. 5B. These results indicate that the paper-based electrochemical DNA biosensor has potential for detecting HPV type 16 DNA in PCR samples in clinical samples in future work.

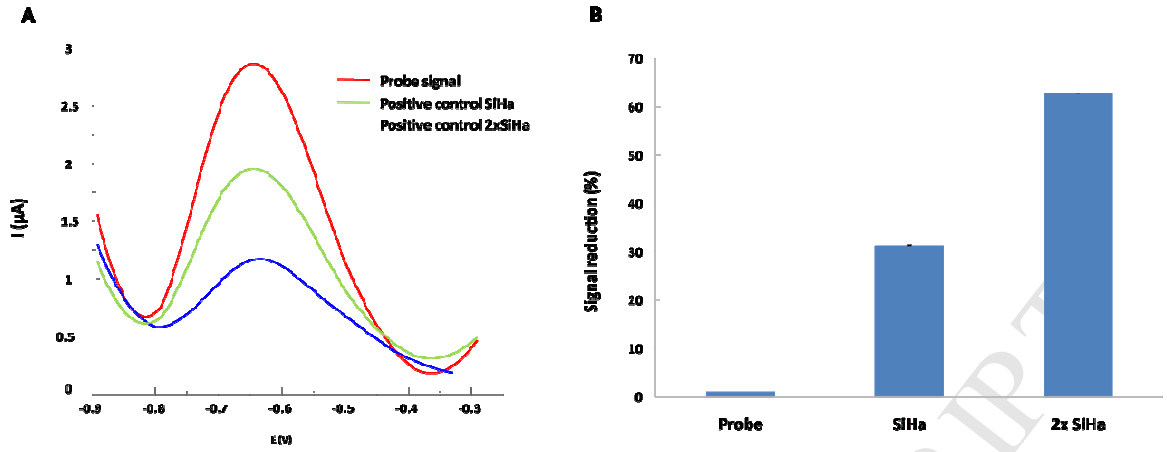


Fig 5. Square-wave voltammograms of AQ-PNA/G-PANI/SPCE probe in the presence of PCR-amplified HPV type 16 positive sample from SiHa cell-line.

4. Conclusions

A novel paper-based electrochemical DNA biosensor was developed and used for the determination of high-risk HPV type 16 using AQ-PNA probe immobilized on a G-PANI/SPCE modified electrode. The AQ-PNA probe was modified with negatively charged amino acids at the C-terminus to enable electrostatic immobilization on the cationic G-PANI electrode. Using SWV, the electrochemical current decreased after hybridization with the complementary target DNA. Under optimal conditions, a linear range of 10-200 nM was obtained and the limit of detection was determined to be 2.3 nM. The proposed DNA sensor also exhibited very high selectivity against non-complementary 14-base oligonucleotides, including HPV types 18, 31 and 33 DNA. Finally, this sensing system was successfully applied to detect the PCR amplified DNA from HPV type 16 positive SiHa cells. As was demonstrated, several features make this highly sensitive ePAD DNA biosensor suited as an alternative tool for the diagnostic screening and detection of cervical cancer. First, the ePAD can provide a low-cost, disposable sensor for this POC application. Second, the immobilization through electrostatic attraction using G-PANI modified electrode is attractive relative to covalent immobilization because of its inherent simplicity. Third, the inkjet printing

418 method for electrode modification process provides the potential for mass production while helping
419 to reduce the variation among individual sensors, which is crucial for disposable sensor.
420
421 **Acknowledgments**
422 The authors gratefully appreciate the financial supports from Thailand Graduate Institute of
423 Science and Technology (TG-44-09-55-014D), the 90th Anniversary of Chulalongkorn University
424 Fund (GCUGR1125582020D) and the Thailand Research Fund (DPG5780002). Also, this research
425 was funded by the Ratchadapisek Sompoch Endowment Fund (2016), Chulalongkorn University
426 (CU-59-008-HR). CSH acknowledges funding from Colorado State University.
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563

Highlights

- A paper-based DNA biosensor using AQ-PNA probe and G-PANI modified electrode was first developed.
- This developed DNA biosensor was highly specific over the non-complementary DNA.
- This sensor was successfully applied to detect the HPV-DNA type 16 obtained from cancer cell lines.
- This sensor is inexpensive and disposable, which can be incinerated easily and safely after use.