Electrochemical DNA biosensor for the multianalyte detection of HPV-16 and HPV-18 in oral and cervical lesions

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Abstract

Infection with high-risk human papillomavirus (HPV) is a major risk factor for oral and cervical cancers. In this study, we developed an electrochemical DNA biosensor for detection of HPV-16 and HPV-18, which are the 2 most prevalent genotypes for development of oral and cervical cancers. The assay involves the sandwich hybridization of the HPV target to silica-redox dye reporter probe and capture probe, followed by electrochemical detection. The sensor was found to be highly specific and sensitive, with detection limit of 22 fM for HPV-16 and 20 fM for HPV-18, between the range of 1 fM to 1 μ M. Evaluation with oral and cervical samples showed that the biosensor result was consistent with the nested PCR /gel electrophoresis detection. The biosensor assay could be completed within 90 minutes. Due to its simplicity, rapidity and high sensitivity, this biosensor could be used as an alternative method for HPV detection in clinical laboratories. [151 words]

Introduction

Oral cancer is one of the most common and considered the sixth most widespread cancer worldwide ¹. More than 90% of all oral cancers are oral squamous cell carcinomas (OSCC). OSCC develops from transformed keratinocytes of the oral epithelium, which had accumulated multiple genetic mutations, causing them to become cancerous ². Tobacco, betel quid chewing, alcohol consumption, smoking and human papillomavirus (HPV) infections are considered to be the most critical risk factors for OSCC ^{3,4}.

Similarly for cervical cancers, HPV has been well established as the main risk factor. HPV are non-enveloped, double-stranded DNA viruses from the Papillomaviridae family that infect the mucosal and cutaneous epithelia. The International Agency of Research of Cancer (IARC) has declared that HPV-16, followed by HPV-18, were among the most common 'high-risk' genotypes due to their oncogenic potential. These HPV genotypes have been linked to OSCC, oropharyngeal squamous cell carcinomas (OPSCC) ⁵⁻¹¹ and cervical lesions.

Identification of HPV in oral cancers is important as the clinical outcome and prognosis may differ from HPV-negative cases ¹². There are currently no standardized or FDA-approved screening methods for detection of oral cancer ¹³. Diagnosis is done by physical assessment and microscopic examination of tissues or cells. A screening tool that could give early detection of HPV presence in oral samples is crucial for appropriate diagnosis and prompt treatment. Genotyping to identify HPV-16 and -18 has been recommended as a screening tool for cervical cancer for patients who have normal cytology before going for colposcopy ¹⁴. Several studies have observed co-infection of HPV-16/-18 with other HPV types and this may modify the risk of cervical cancer ^{10,15}.

Current methods for HPV detection include cytology, histopathology, immunohistochemistry, polymerase chain reaction (PCR) and quantitative reverse transcription-PCR (qRT-PCR) ⁸. The sensitivities of cytological tests vary greatly according to the experience of the cytologists and the type of quality control in place ¹⁶. More recently, several commercially available HPV tests based on PCR, could also provide genotyping information, usually for the oncogenic, high-risk genotypes such as HPV-16 and HPV-18. HPV genotyping could provide additional information for risk assessment, prognosis and therapy, such as the use of therapeutic vaccines that target specific HPV genotypes ¹¹. Electrochemical DNA biosensors have been developed as a rapid, portable, economical, sensitive and specific detection tool for various targets. Many DNA biosensors have been developed for HPV detection, mainly focusing on cervical cancer samples ¹⁷⁻²⁴. To the best of our knowledge, this is the first report of an electrochemical DNA biosensor for the multianalyte detection of HPV-16 and HPV-18 from both oral and cervical cancer samples. Our biosensor platform uses silica nanoparticles loaded with 2 different redox indicators and magnetic bead-capture probes that specifically target the HPV E6/ E7 oncogenes of HPV-16 and HPV-18. The analytical performance of the developed biosensor was compared with the result of nested PCR / gel electrophoresis.

Results And Discussion

Characterization of the silica nanoparticles (SiNPs) and silica redox-active dye (Si-dye)

The redox-active dyes, methylene blue (MB) and acridine orange (AO), were loaded into the silica nanoparticles through surface-reactive functional groups. This method ensured that the leakage of dye molecules from the silica particles was negligible, due to the strong electrostatic attractions between the positively charged (cationic dyes) and the negatively charged silica ^{25,26}. Fig.1 shows the size distribution, scanning electron microscopy (SEM) image and differential pulse voltammetry (DPV) peaks of SiNPs (without dye), SiMB and SiAO. The core nanoparticle, SiNPs, had a mean diameter of 466.31 ± 7.62 nm. The size increased to 506.45 ± 3.98 nm and 518.57 ± 4.21 nm after deposition of the MB and AO redox dyes, respectively. DPV analysis gave sharp peaks at -0.3 V (MB) and 0.7 V (AO). The current responses of both Si-dyes were similar at 10 mg/mL concentration. The use of MB- and AO-doped silica nanoparticles for single and multiplex target detection in electrochemical biosensor assays have been previously described ^{25,27}.

A layer-by-layer procedure was used to create two oppositely-charged bilayers on the Si-dye, starting with the deposition of positive-charged PAA and followed by the negative-charged PSS. This enables avidin, which has a strong positive charge, to form an ionic interaction with the PSS layer. Thus, the binding of the biotin-labelled reporter probe to avidin could be achieved.

Optimization of the assay conditions

The sandwich hybridization parameters such as amount of capture probe-magnetic bead (CP-MNB), reporter probe-conjugated to Si-dye/Avidin (Si-RP) and hybridization time were optimized using 1 pM of complementary LT of each genotype or non-complementary target (non-HPV viruses). The optimization was performed to maximize the differences between positive and negative signals and decrease nonspecific binding. Statistical analysis was used to determine the best assay conditions. Fig. 2 shows the result of the optimization experiments. The best sensor performance was achieved with 2.0 μ L of CP-MNB (Fig.

2a), 20 μ L of Si-RP (Fig. 2b) and 30 min hybridization time (Fig. 2c). At the same concentration of 1 pM LT, the current signals for both genotypes were quite similar throughout most of the conditions tested in this experiment.

Analytical performance of the assay

The analytical performance of the biosensor was performed using the optimized experimental conditions. The specificity of the assay was evaluated using 1 pM HPV16 complementary, 1 base and 3 bases mismatch and non-complementary LT (Hepatitis A virus, HAV; Hepatitis B virus, HBV; Hepatitis C virus, HCV; and Hepatitis E virus, HEV). A current signal that was equal or greater than +3 standard deviations (3 SD) above the mean of the blank (background) signal was considered a positive result. All reactions that contain the complementary target yielded positive signal, while the non-complementary and mismatch targets were all negative (Fig. 3a). Moreover, the assay could discriminate between perfectly complementary targets and one or three bases mismatch targets, demonstrating the highly specific nature of the assay. Analysis with showed that the signals from blank, non-complementary and mismatch targets were significantly different from the complementary targets.

The analytical sensitivity was determined using varying concentrations of complementary LT, from 1 fM to 1 μ M. The calibration curve for both HPV-16 and HPV-18 showed a linear relationship with correlation coefficient of 0.9912 between 1 fM to 1 μ M (Fig. 3b). The limits of detection (LOD) of HPV-16 and HPV-18 were 22 fM and 20 fM, respectively. The corresponding voltammograms show the current response increases as the concentration of LT increases (Fig. 3c, d).

It is interesting to note that the LOD for both genotypes were similar, even though 2 different redox indicators were used. This could be due to both MB and AO having similar electrochemical response on SPCE. This similarity in detection limit was also seen in our previous study where we used Si-MB and Si-AO for multiplex detection of 2 different genes²⁸. The analytical performance of the biosensor was compared with previously published DNA biosensors for HPV-16 and HPV-18 (Table 2). Our biosensor showed comparable results in terms of the limit of detection when compared to similar studies that utilized nanomaterials with electrochemical detection for HPV-16/18^{19,29}.

Table 2. Comparison of analytical sensitivity of other previous reported HPV-16/HPV-18 electrochemical DNA biosensors.

Sensor platform	Technique	Target	Detection limit	Ref.
Reduction signals of methylene blue on L-cysteine film modified gold electrode surface	Differential pulse voltammetry	HPV-16	18.13 nM	29
Immobilized anthraquinone-labeled pyrrolidinyI peptide nucleic acid probe on chitosan-modified disposable SPCE	Square-wave voltammetry	HPV-16	4 nM	30
Potential relaxation of the double layer of a DNA-modified DNA-modified gold electrode	Electrochemical impedance spectroscopy	HPV-16	0.38 nM	17
Detection of loop-mediated amplification (LAMP) products on carbon-based electrode chips with chronoamperometric monitoring of benzoquinone reduction	Chronoamperometry	HPV-16	0.1 ng	31
Modified indium tin oxide electrode coated with chitosan capped gold nanoparticles	Square wave voltammetry	HPV-16	1 pM	23
DNA hybridization on interdigitated electrode chips surface modified with gold doped zinc oxide nanorods	Electrochemical impedance spectroscopy	HPV-16	1 fM	20
Interdigitated electrodes modified with titanium dioxide nanoparticles	Unspecified voltammetric technique	HPV-16	0.1 fM	32
Carbodiimidazole-modified interdigitated electrode with reporter conjugated gold nanorod	Unspecified voltammetric technique	HPV-16	1 aM	33
Reduced graphene oxide and multiwalled carbon nanotubes with L-cysteine functionalized gold nanoparticles on SPCE	Differential pulse voltammetry	HPV-18	0.05 fM	19
Gold electrodes co-immobilized with thiolated probe and bipodal alkanethiol with horseradish peroxidase-labelled reporter probe	Chronoamperometry	HPV-16 HPV-18	220 pM 170 pM	34
Electrochemical signal of silica-methylene blue (HPV-16) and silica-acridine orange (HPV-18) reporter probes on SPCE	Differential pulse voltammetry	HPV-16 HPV-18	20 fM 22 fM	This study

A total of 55 oral samples and 25 cervical cancer samples were used in this evaluation. The nested PCR amplicons were detected using the biosensor assay and the result was compared with agarose gel electrophoresis. For the electrochemical detection, a current signal that was equal or greater than +3 standard deviations (3 S.D.) above the mean of the blank (background) signal was considered as positive. Fig. 4 shows the result of HPV-16 and HPV-18 detection in the clinical samples. Two of the 13 OSCC samples were positive for HPV-16 and HPV-18, respectively. All the other oral samples were negative. For the cervical cancer samples, 10 were positive for HPV-16 only, 5 were positive for HPV-18 only and 6 were positive for both HPV-16 and HPV-18. The comparison between biosensor and agarose gel electrophoresis results was 100% concordant. The result is summarized in Table 3.

In this study, 6 cervical samples were found to harbor both types of HPV. Several studies have shown that more than one type of HPV could be detected in oral³⁵ and cervical^{21,31} samples. The whole assay, starting from target hybridization to electrochemical measurement could be completed in 90 min. This method could be used as an adjunct to the available screening tools for detection of oral and cervical cancers. Furthermore, this biosensor could potentially be used for genotyping in other clinical samples as well.

Table 3. Comparison of HPV-16 and HPV-18 detection in clinical samples using gel electrophoresis and electrochemical biosensor.

Clinical samples	No. of sample	HPV-16		HPV-18	
		Result		Result	
		Gel electrophoresis	Biosensor	Gel electrophoresis	Biosensor
WBC DNA	1	0	0	0	0
SiHa cell	1	1	1	-	-
HeLa cell	1	0	-	1	1
Normal mucosa	14	0	0	0	0
Oral lichen planus	25	0	0	0	0
Oral leukoplakia	3	0	0	0	0
OSCC	13	1	1	1	1
Cervical lesions ^{*a}	25	16	16	11	11

^{*a}: Cervical lesion sample result (below table)

Assay	Samples	CxCA1	CxCA2	CxCA3	CxCA4	CxCA5	CxCA6	CxCA7	CxCA8	CxCA9	CxCA10	CxCA11	CxCA12	CxCA13	CxCA14	CxCA15	CxCA16	CxCA17	CxCA18	CxCA19	CxCA20	CxCA21	CxCA22	CxCA23	CxCA24	CxCA25
		HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16	HPV18	HPV16
Nested PCR	HPV16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HPV18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Biosensors	HPV16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HPV18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Conclusion

An electrochemical biosensor for two high-risk HPV genotypes (HPV-16 and HPV-18) has been successfully developed. The biosensor shows high sensitivity and specificity for the HPV-16 and -18 genotypes. Detection of these 2 genotypes in oral and cervical samples yielded good results. Therefore, the biosensor could be used as a screening tool for accurate detection of these oncogenic HPV types, which will certainly help in the diagnosis and prognosis of cancer.

Methods

Chemicals, kits and instrumentation

All buffer solutions were prepared with purified milli-Q water (18.2 MΩ). Poly (allylamine hydrochloride), (PAA MW~50,000), poly (sodium 4-styrenesulfonate) (PSS, MW~70,000), tetraethyl orthosilicate (TEOS), acridine orange (AO) and avidin from egg white were purchased from Sigma-Aldrich (USA). Sodium chloride, methylene blue (MB), 2-propanol, 25% ammonium solution, 28-30% ammonium hydroxide, acetone and 95% ethanol were purchased from Merck (Germany). Dynabeads™ MyOne™ Streptavidin T1 was purchased from Thermo Fisher Scientific (USA). AccuPower 2X Greenstar qPCR Master Mix was purchased from Bioneer (Korea), 2x PCR Master Mix Solution (i-StarTaq) was purchased from iNtRON Biotechnology (Korea).

For electrochemical measurements, screen-printed carbon electrodes (SPCE) were obtained from Quasense (Thailand) and the PalmSens4 potentiostat with PStace software version 5.8 was obtained from Palmsens (The Netherlands).

Design of primers and probes

Nested PCR primers for HPV 16 and HPV-18 were designed based on the E6/ E7 HPV oncogenes as described in previous studies ^{35,36}. The probes were designed to bind to the PCR amplicons and tagged with a biotin moiety at either the 3' or the 5' end. The mismatched linear targets (mLT) contain 1 or 3 mismatched bases. The oligonucleotide sequences are listed in Table 1. PCR primers were purchased from Bionics (Korea), while probes and DNA linear targets (LT) were purchased from Integrated DNA Technologies Pte. Ltd., (Singapore).

Table 1. The nested PCR primers, probes and DNA linear targets used in this study.

Type	Primer name	Sequence (5' à 3')
HPV-16 Outer primer	GP-E6-3F	GGGWGKKA CTGAAATCGGT
	GP-E6-5B	CTGAGCTGTCA RNTAATTGCTCA
	GP-E6-6B	TCCTCTGAGTYGYCTAATTGCTC
HPV-16 Inner primer	Forward	CACAGTTATGCACAGAGCTGC
	Reverse	CATATATTCATGCAATGTAGGTGT
HPV-16 Probes and linear targets	HPV-16-CP	Biotin-GTGTG TACTGCAAGCAAC
	HPV-16-RP	TGCGACGTGAGGTATATG-Biotin
	HPV-16-LT	ATCCCGAAAAGCAAAGTCATATACCTCACGTCGCAGTAACTGTTGCTT GCAGTACACACATTCTAATATTATATATGTA TAGTTGTTT
	HPV-16-1mLT	AGTCATATACCTCACGTCGCAGTAACTGTTGCTTGCG <u>G</u> GTACACACATT
	HPV-16-3mLT	AGTCATATACCTCACGTCGCAGTAACTGTTGCTTGCG <u>GAC</u> ACACACATT
HPV-18 Outer primer	Forward	TGAAATTCGGTTGACCTTC
	Reverse	GGTCGTCTGCTGAGCTTTCT
HPV-18 Inner primer	Forward	ATGTCACGAGCAATTAAGC
	Reverse	TTCTGGCTTCACACTTACAACA
HPV-18 Probes and linear targets	HPV-18-CP	Biotin-CCACAACGTCACACAATG
	HPV-18-RP	GTGTTGTAA GTGTGAAGC-Biotin
	HPV-18-LT	TTCTGGCTTCACACTTACAACACATACACAACATTGTGTGACGTTGTG GTT CGGCTCGT CGGGCTGGTAAATGTTGATGATTAACTG
	HPV-18-1mLT	CTGGCTTCACACTTACAACACATACACAACATTGTGTG <u>G</u> CGTTGTGGT TCGGCT
	HPV-18-3mLT	CTGGCTTCACACTTACAACACATACACAACATTGTG <u>CAG</u> CGTTGTGGT TCGGCT
HPV 16 /18 Blocking probe	dT10_BP	TTTTTTTTTT-Biotin
Non-complementary linear target	HAV-LT	CGTTGAATGGTTTTTGTCTTAACAACTACCAATATCCGCCGCTGTTACC
	HBV-LT	AAGATGTTGTACAGACTTGGCCCCAATACCACATCATCCATATAACTGA AAGCCAA
	HCV-LT	CTGCGTGAAGACAGTAGTTCCTCACAGGGGAGTGATTCATGGTGGAGTGT CGCCC
	HEV-LT	GGATTGCGAAGGGCTGAGAATCAACCCGGTCACCCAGAAACCACCGCC GG

(CP: capture probe, RP: reporter probe, LT: linear target, mLT: mismatched linear target, BP: blocking probe. Underlined base(s) indicate the mismatched sequence)

Silica seed nanoparticles (SiNPs) were synthesized and the redox active dye was incorporated into the SiNPs by a modified method described previously²⁶. Firstly, 1.375 mL of TEOS was added to 9.6 mL of 2-propanol under slow stirring. A mixture of 0.5 mL of 25% (v/v) ammonia and 1.025 mL of milliQ-water were added with constant stirring for 1 h, the solution was heated to 50°C to produce silica seed particles. Silica seed solution was added to 5 mL of TEOS, 227.5 mL of 2-propanol and 44.5 mL of 8.29 % (v/v) ammonia solution. Then, 91.25 mL of TEOS was added to the mixture at a rate of 0.5 mL/min, and the reaction was allowed to continue for an additional 1 h with fast stirring under 50°C. Finally, the silica microsphere particles were isolated by centrifugation at 10,000 rpm for 10 min. The pellet was washed with milliQ-water and centrifuged at 8,000 rpm for 5 min. The washing step was repeated 4 times. The silica pellet was obtained by drying at 105°C in an oven.

Loading of SiNPs with redox-active dye

Firstly 0.3 g of silica powder was added into 10.9 mL of 2-propanol containing 1.5×10^{-5} mol of a redox dye (MB for HPV-16 and AO for HPV-18) and 0.55 mL of TEOS were mixed by sonication until it formed a colloidal suspension. After that, a separate solution of 25% ammonia was diluted with 12 mL of milliQ-water was added into the mixture. The reaction was allowed to proceed for 2h at 40°C. The pellet was washed with milliQ-water and centrifuged at 8,000 rpm for 5 min. This step was repeated 4 times and dried at 105°C in an oven. SiNPs and dye incorporated silica (Si-dye) were characterized by scanning electron microscope, SEM (JMS-6610LV by JEOL Ltd., Tokyo, Japan).

Preparation of the reporter probe

Ten mg/mL silica redox-active dye (Si-dye) was sequentially incubated in 0.2 mL of PAA and PSS polyelectrolytes (10mg/mL in 0.5 M NaCl) at room temperature for 30 min, respectively. After washing to remove the excess of polyelectrolytes the particles were dispersed in 1 mL of 10 mM PB (pH7.0). Next, 10 µL of avidin (21.14 mg/mL) was added into the solution and incubated at 37°C for 1 h 30 min. The avidin-coated Si-dye/PAA-PSS (referred to as Si-dye/Avidin) particles were resuspended in 2 mL of 0.1 M PBS (pH 7.0). The conjugation of Si-Avidin with the reporter probe (RP) was achieved by mixing 0.1 mL of Si-Avidin with 1 mL of 10 mM RP and 99 mL of 10 mM blocking probe (BP). Both RP and BP contain a biotin moiety at the 3' end, which can bind to avidin. HPV-16-RP was conjugated with Si-MB/Avidin and HPV-18-RP was conjugated with Si-AO/Avidin. The mixture was incubated at room temperature for 30 min. The RP-conjugated to Si-dye/Avidin (referred to as Si-RP) was resuspended with 250 mL of 1 M PBS and kept at 4°C until use.

Preparation of capture probe

Four µL of 100 µM capture probe (CP), 12 µL of 100 µM blocking probe and 100 µL of Dynabeads™ MyOne™ Streptavidin T1 (MNB, 10 µg/µL) were mixed and allowed to incubate at room temperature for 30 min with gentle agitation. The CP-MNB were separated from the unbound CP by magnetic separation, followed by washing step for 3 times with 20 mM PBS (pH 7.0). Finally, the CP-MNB was resuspended in 100 mL of 20 mM PBS and stored at 4°C until use.

Target hybridization and signal detection

First, 18 µL of DNA target was mixed with 2 µL of CP-MNB and incubated at 50°C for 30 min. The separation of DNA target bound to CP-MNB (referred to as CP-MNB-Target) from the unbound CP-MNB was performed by magnetic separation, followed by washing step. Next, 20 mL of Si-RP was added to the CP-MNB-Target mixture and incubated at 50°C for 30 min to allow the sandwich hybridization to occur. After that, the separation of bound and unbound targets was facilitated by magnetic separation. Finally, the pellet was re-suspended with 0.1 M PB containing potassium chloride (PB/KCl) (pH 7.0). This solution was pipetted onto the SPCE. Differential pulse voltammetry (DPV) was performed by scanning from -0.5 to -0.1 V (for HPV-16) and 0.2 to 1.0 V (for HPV-18), with a step potential of 0.01 V, time equilibration of 150 sec, modulation amplitude of 0.05 V with an interval time of 0.05 sec, and 0.1V/s scan rate.

Sensitivity and specificity of the assay

The sensitivity of the assay was evaluated using 1 fM to 1 µM of DNA linear targets (HPV-16-LT and HPV-18-LT). The specificity of the assay was evaluated using 1 pM of DNA linear target. Complementary targets (HPV-16-LT and HPV-18-LT), mismatched targets (HPV-16-1mLT, HPV-16-3mLT, HPV-18-1mLT, HPV-18-3mLT) and non-complementary targets, which consisted of DNA sequences of other viruses such as Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV) and Hepatitis E virus (HEV).

Extraction of genomic DNA from clinical samples

Forty oral samples consisting of 25 oral lichen planus (OLP), 3 oral leukoplakia (OL), 13 OSCC and 14 normal mucosa, and 17 cervical tissue samples were used in this study. Oral epithelial cells were collected using soft toothbrush from the buccal mucosa. DNA extraction was performed using Puregene® Buccal Cell Core Kit A (Qiagen®, Germany) according to the manufacturer's protocol. DNA from cervical brushes were extracted using QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. HeLa cell line containing HPV18 and SiHa cell line containing HPV16 were used as HPV positive controls, while human white blood cells were used as negative control. Plasmids containing cloned genome of HPV-16 and genomes of both HPV-16 and HPV-18 (referred to as mixed HPV-16/18) were also used as positive controls. The plasmids were kindly provided by Prof. Chamsai Pientong, Faculty of Medicine, Khon Kaen University, Thailand.

Nested PCR and detection of HPV-16 and HPV-18 from clinical samples

HPV-16 and HPV-18 typing were performed using nested PCR³⁷ with the HPV-16 and HPV-18 outer and inner primer sets shown in Table 1. The nested PCR mixture contained 1x of PCR Master Mix Solution, 0.3 µM of primers and 50 ng of DNA template in a total volume of 25 µL. Reaction lacking DNA template and non-HPV DNA (WBC) were used as negative controls, while plasmids containing HPV-16 genome and mixed HPV-16/-18, and HeLa cell DNA were assigned as

positive controls. First round of nested PCR was performed in a thermal cycler with the cycling parameters as follows: For HPV-16: 94 °C for 4 min, followed by 40 cycles (1 min at 94 °C, 1 min at 40 °C and 1 min at 72 °C), with a final extension at 72 °C for 10 min; For HPV-18: 95 °C for 4 min, followed by 35 cycles (30 sec at 95 °C, 30 sec at 61 °C and 30 sec at 72 °C), with a final extension at 72 °C for 1 min.

One µL of the first round (outer) PCR product was used as a template for second round (inner) PCR. The thermal cycling conditions for second round PCR as follows: For HPV-16: 94 °C for 4 min, followed by 35 cycles (30 sec at 94 °C, 30 sec at 56 °C and 45 sec at 72 °C), with a final extension at 72 °C for 4 min; For HPV-18: 95 °C for 4 min, followed by 35 cycles (30 sec at 95 °C, 30 sec at 63 °C and 30 sec at 72 °C), with a final extension at 72 °C for 1 min. The PCR product was detected using electrochemical biosensor and the result was compared with agarose gel electrophoresis. Briefly, 18 µL of 50 ng nested PCR product was mixed with 2 µL of CP-MNB. Hybridization and electrochemical signal detection were performed as previously described above.

Ethical consideration

The study involving the oral samples was reviewed and approved by the Ethics Committees of the Faculty of Dentistry and Faculty of Pharmacology, Mahidol University, Thailand (COA.No.MU-DT/PY-IRB 2020/008.1701 on Jan 17, 2020), while the study involving the use of cervical samples was reviewed and approved by the Ethics Committees of Khon Kaen University (HE562296) and Ubonratchathani Cancer H, Thailand (EC012/2013). The institutional review board of the Ethics Committee for human research waived the need for consent because all samples were anonymous.

Statistical analysis

Statistical analysis was performed using SPSS version 18.0. All electrochemical measurements were performed on 5 independent SPCEs, and the current responses are presented as mean values $\pm$ standard deviation (SD). The ANOVA test was used for the analysis, with the p-value of less than 0.05 was considered statistically significant.

Declarations

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Competing interests

The authors declare no competing interests.

Author contributions

TC, LSY, WB and BL contributed to designing the study, writing and editing the manuscript. TC, PT PC and SJ collected the clinical samples and TC and PC performed the PCR analysis. TC performed the electrochemical experiments. TC, LSY and BL analyzed data and prepared figures. CP, WB, PJ and BL conceived and supervised the study and wrote the manuscript. All authors participated in manuscript editing and approved the manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Characterization of the silica nanoparticles and Si-dye. Size distribution of SiNPs (a), SiMB (b) and SiAO (c), with SEM images (see inset). (d) Differential pulse voltammogram showing the peak potentials of SiNPs coated with a redox-dye layer (black line=SiNPs, blue line=SiMB and orange line=SiAO). (e) Current responses of 10 mg/mL of SiMB (blue bar graph) and SiAO (orange bar graph). The current signals presented as mean values ± standard deviation (SD) from electrochemical measurements (n = 5).

Figure 2

Optimization of the assay conditions. The effect of varying the amount of CP-MNB (a), Si-RP (b) and hybridization time (c) on the current responses using 1 pM LT of each genotype. The conditions that gave the best signals for both HPV-16 and HPV-18 are highlighted in yellow. All current responses are presented as mean values $\pm$ standard deviation (SD), $n = 5$.

Figure 3

Assay performance. (a) Specificity of the assay for HPV-16 (blue bar) and HPV-18 (orange bar), tested with 1 pM complementary targets (HPV16-LT and HPV18-LT), 1 pM mismatch DNA targets (HPV16-1mLT, HPV16-3mLT, HPV18-1mLT, HPV18-3mLT), and 1 pM non-complementary targets (HAV-LT, HBV-LT, HCV-LT, HEV-LT). (b) Sensitivity of the assay for HPV-16 (orange triangle) and HPV-18 (blue square) tested with their respective complementary linear targets. Differential pulse voltammograms showing the increase in the current signal as the concentrations of the HPV-16 (c) and HPV-18 (d) linear targets increased. Dashed lines show the blank signal. All current responses are presented as mean values $\pm$ standard deviation (SD), $n = 5$.

Figure 4