Development of Biosensor for High Risk HPV Detection in Cervical Cancer

การพัฒนา Biosensor สำหรับการตรวจหาชนิดของเชื้อ Humanpapillomavirus ในผู้ป่วยมะเร็งปากมดลูก

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ABSTRACT

Persistence infection with high risk Human papilloma virus (HR-HPV) has been implicated as one of the major risk factor of cervical cancer development. In this study, we establish a DNA piezoelectric biosensor to detect 11 HR-HPV (type 16, 18, 45, 33, 39, 51, 52, 56, 58, 59 and 68) Biotinylated detection probe was attached on gold electrode 9 MHz Quartz crystal microbalance (QCM) using biotin-avidin linking system. Target DNA of 11 HR-HPV previously amplified with specific primers overhanging with detection probe sequence at their 5' end were tested with our developed QCM biosensor. The sensitivity of detection on QCM sensor was comparable to the conventional agarose gel electrophoresis. Our QCM prototype demonstrates good precision with %CV of 6.5. In conclusion, QCM sensor can detect all 11 HR-HPV which isolated from cervical cancer tissues. The sensor should be further developed in couple with PCR for complete HPV detection. This sensor might be exerted as a new tool for fast HR-HPV screening in population based analysis.

บทคัดย่อ

สาเหตุของการเกิดมะเร็งปากมดลูกนั้น เกิดจากการติดเชื้อ Human papilloma virus (HPV) ชนิดที่เสี่ยงสูง ในการศึกษาครั้งนี้อาศัยหลักการเครื่องมือตรวจวัดน้ำหนักระดับนาโนกรัมหรือ Quartz crystal microbalance

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(QCM) เพื่อตรวจหา ดีเอ็นเอของเชื้อ HPV ในชนิดที่เสี่ยงสูงจำนวน 11 ชนิดได้แก่ ชนิด16, 18, 45, 33, 39, 51, 52, 56, 58, 59 และ68 โดยใช้โปรตีนไบโอตินที่ติดกับ Detection probe นำมาติดอยู่บน QCM ขนาด 9 MHz สำหรับเป็นตัวตรวจจับดีเอ็นเอของเชื้อ HPV ซึ่งได้จากวิธี Polymerase chain reaction (PCR) ด้วยไพรเมอร์ที่จำเพาะกับเชื้อ HPV แต่ละชนิดโดยติด Detection sequence ที่ด้าน 5' ของไพรเมอร์ดังกล่าว เพื่อให้ PCR ผลผลิตสามารถจับได้กับ Detection probe บน QCM sensor ผลการทดลองพบว่าความไวของการตรวจวัดผลผลิต DNA ด้วย QCM sensor เท่ากับการตรวจวัดด้วยวิธี agarose gel electrophoresis และความแม่นยำของการตรวจวัด (%CV) เท่ากับ 6.5 % และผลการตรวจหาผลผลิต DNA โดยวิธี PCR ของเชื้อ HPV ชนิดเสี่ยงสูงจากผู้ป่วยมะเร็งปากมดลูก พบว่าให้ผลสอดคล้องกับการตรวจหา ผลผลิต DNA ด้วย agarose gel electrophoresis ซึ่งหลักการดังกล่าวสามารถพัฒนาไปสู่การทำ PCR และ QCM บนชิบและเป็นเครื่องมือหนึ่งสำหรับตรวจผู้ป่วยต่อไป

Key Words : Quartz crystal microbalance, Human papillomaviruses, Cervical cancer, QCM Sensor
คำสำคัญ : เครื่องมือตรวจวัดน้ำหนักระดับนาโนกรัม, มะเร็งปากมดลูก

Introduction

Infection with human papillomaviruses (HPVs) can cause warts on cutaneous epithelium, while in the anogenital region, these viruses can cause both genital warts and various forms of cancer in men and women (Bernard 2005). The main interest in HPV related cervical cancer is the annual incidence of almost half a million as well as a mortality rate of approximately 50% (Parkin, Bray et al. 2001). Persistent HPV infection of the cervical epithelium is considered to be a major risk factor for the development of cervical cancer. Abnormal cervical epithelial cells can be detected microscopically by Papanicolaou (Pap) staining of conventional cervical smears. Test evaluation for cytology was described by many researchers with various sensitivity from 44% to 78% and the specificity from 91% to 96% . Even though pap smear is used for cervical screening programmers to detect women at risk of disease progression, molecular detection of HPV provides as an alternative approach to screening and patient management in modern laboratory (Cuschieri and Cubie 2005).

At present, 118 HPV genotypes have been classified according to their biological niche, oncogenic potential and phylogenetic position (de Villiers, Fauquet et al. 2004). HPV types are distinguished to high-risk (HR) and low-risk (LR) depending on their association with cervical carcinoma or associated precursor lesions. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56,58, 59, 68, 73 and 82 are considered oncogenic or high-risk types (Munoz, Bosch et al. 2003). Identification of HPV genotypes by PCR has been claimed to be a very sensitive and specific method (Molijn, Kleter et al. 2005). However, the screening is practically limited in large scale population. Moreover, DNA staining with ethidium bromide is carcinogenic (Tombelli, Mascini et al. 2000). Recently, there has been increasing interest in biosensor technology for designing micro device

for DNA fragment detection that can be integrated to PCR. Among these devices, QCM (Quart crystal microbalance) microdevice - based assay for HPV genotypes are a promising candidate for the detection of PCR product (Del'Atti, Zavaglia et al. 2007). The objective of this study is to develop and evaluate biosensor prototype for detection of high-risk HPV types. According to its feasibility to manipulate and rapid detection for large scale population, therefore the development of biosensor based technology for high risk HPV genotype detection will be provided as a potential tool in both screening and diagnosis.

Materials and methods

Materials

All chemicals and reagents used in this study are analytical or molecular biological grade.

Preparation of piezoelectric biosensor

AT-cut, 9 MHz quartz crystals (diameter = 1-inch) with polished Au electrodes (Au area = 1.34 cm2, thickness = 185 microns) coated on both sides were obtained from MAXTEK, INC (USA). Before using, the, gold electrode surface as cleaned with piranha solution (30%,v/v, H_2O_2:H_2SO_4 = 1:3)(F. Caruso 1995) for 2 minutes. The crystals were then washed with distilled water and used immediately afterwards. The initial resonance frequency (f0) was recorded as the baseline after air-dried. The quartz crystal was soaked into the optimal concentration of 3-mercaptopropionic acid (MPA) at room temperature for 1 hour, rinse with ethyl alcohol and distilled water, and allowed to dry. To activate the monolayer, 60 μL of 200 mM 1-ethyl-3 (3-dimethylaminopropil) carbodiimide (EDC) was placed on the surface followed by adding 60 μL of 50 mM *N*-hydroxysulfosuccinimide (NHS).These solutions were left to react on the surface to interact with MPA monolayer at room temperature for 30 minutes. The surface was then rinsed with water and air-dried. Aliquot of 200 μL of the optimal concentration of avidin was placed on the electrode surface at room temperature for 1 hour. The surface was rinsed with water and air-dried. The resonant frequency was measured (f1) again. After that, the quartz crystal was then exposed to a 1 mM ethanolamine at room temperature for 30 minutes, washed the excess ethanolamine out with distilled water and immobilization buffer. Approximately 100 μL of biotinylated detection probes was added to react with avidin layer over the electrode surface at room temperature for 20 minutes. After rinsing with immobilization buffer and distilled water, and air-drying, the resonant frequency (f2) was finally measured to determine the attachment of detection probes on the avidin layer of gold electrode. The sequence of detection probe contains an additional 30 bases having unique sequence which unseen in human or viral genome. The double stranded of target DNA harboring detection probe sequence on their 5'end was hybridized on the surface of QCM sensor at room temperature for 20 minutes sensor was washed well with hybridization buffer and distilled water to remove the excess or unbound DNA. The new frequency (f3) was finally measured after air-dried. The frequency difference between the resonance frequencies of the value displayed before and after the hybridization was determined ($\triangle$F = frequency of the immobilization probe on the crystal (f2) minus frequency of the

hybridization reaction (f3), with f2> f 3). A shift in the frequency difference ($\Delta F = f2 - f3$) was related DNA to hybrid with the nucleic acid probe to immobilized on the quartz crystals

Amplification of HR-HPV using nested multiplex PCR (NMPCR).

All DNA were screened for HPV positive using primer specific for L1 consensus region. GAPDH was used as internal control for the presence of qualified template. Samples with positive HPV were subsequently typing for HR-HPV using nested multiplex PCR (Sotlar k 2004).

Ten microliters of the amplified products were analyzed by electrophoresis on 2% agarose gels and stained with ethidium bromide. Plasmid clones containing known HPV 16, 18, 45, 33, 39, 51, 52, 56, 58, 59 and 68 were used as positive controls.

Result

Optimal Conditions for Quartz Crystal Preparation

Firstly, the gold surface was cleaned with piranha solution. The self assembled monolayer of MPA was formed on the gold electrode of quartz crystal. The activation of the MPA modified crystal with EDC and NHS was done and followed by covalent coupling with avidin. The carboxylic end of the MPA was chemically replaced with a good leaving group via the activated *O* - acrylurea intermediate by treating the monolayer with EDC and NHS. The immobilization of the avidin to the activated sulfides monolayer was done by the formation of the covalent amide group between the activated monolayer and the avidin in exchange of the leaving group of the activated sulfide monolayer. The quartz crystal was then immersed in the ethanolamine to convert the residual carboxyl group to β hydroxyethylamide.Biotinylated oligonucleotide was then bound to the avidin surface.

MPA optimization

The optimum amount of MPA on the surface of crystal was determined for the effective immobilization of 0.2 mg/ml avidin. The MPA self assembly monolayer could not investigated due to the small molecular structure of this short chain thiol compound each quartz crystal was soaked into the MPA at concentration of 1, 5, 10, 15, and 20 mM, followed by 200 mM EDC and 50 mM NHS. Then, 0.2 mg/ml of avidin was placed on the surface of the crystal at room temperature for 1 hour. The resonant frequency was measured and calculated for the frequency change from bare gold electrode of quartz crystal. The resonant frequency was plotted as a function of MPA concentration. Each data point represents the mean ± SD (n = 4) crystal with MPA and avidin ($\Delta F = f0 - f1$). The highest frequency change was obtained from MPA at the concentration of 10 mM or higher (shown in Fig 1a).This optimized MPA concentration was then used to modify on the quartz crystal surface for the other immobilization experiments.

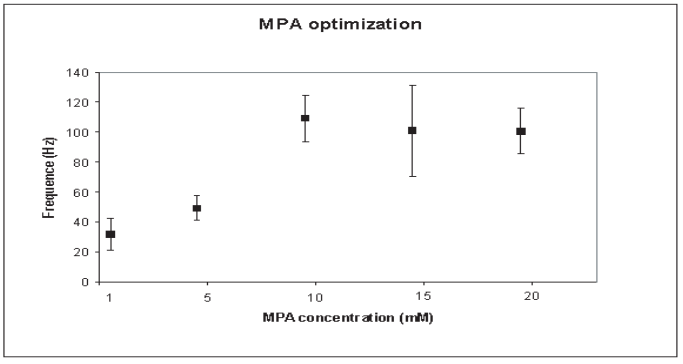
Avidin optimization

Ten mM of MPA was monolayered on the crystal surface. The avidin solution at the concentration 0.125, 0.25, 0.5, 1.0, and 2.0 mg/dl were immobilized on this modified surface at room temperature for 1 hour. The resonant frequency was

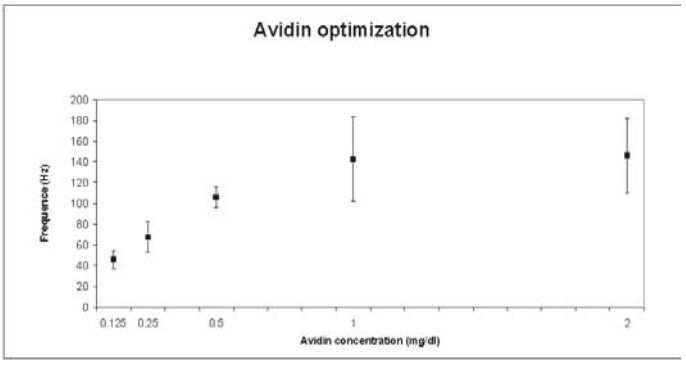
observed and calculated for the frequency change from the original quartz crystal. The resonant frequency was plotted as a function of avidin concentration. These were represented by the frequency differences between the frequency value of after (f1) and before (f0) avidin immobilization ($\Delta F = f0 - f1$). Each data point represents the mean ± SD (n = 3). The results obviously showed that the higher avidin concentration gave the bigger frequency change up to the concentration of 1.0 mg/ml. The Fig 1b also showed the saturation of immobilization when using the concentration of 1.0 mg/ml or higher.

Optimization of the 5′- Biotinylated detection probe Immobilization

The avidin-modified surface of quartz crystal 10 mM of MPA, and 0.1 mg/ml of avidin were immobilized with detection probe at concentration of 0.25, 0.50, 0.75, 1.00, and 1.50 μM at room temperature for 20 minutes. The resonant frequency was observed and calculated for the frequency change from the original crystal. The frequency changes were presented by the frequency differences between the frequency value of biotinylated detection probe immobilized onto the avidin coated on gold electrode (f2) and frequency value of original quartz crystal (f0) before immobilization ($\Delta F = f0 - f2$). Each data point represents the mean ± SD (n = 3). At higher amount detection probe (1.5 μM), the frequency change due to M13 probe immobilization was no longer dependence on the probe amount as shown in Fig1c. The highest frequency change was obtained from 1.0 μM of detection probe. Therefore, 1.0 μM of detection probe was chosen to obtain the maximum frequency change in the experiment.



a.



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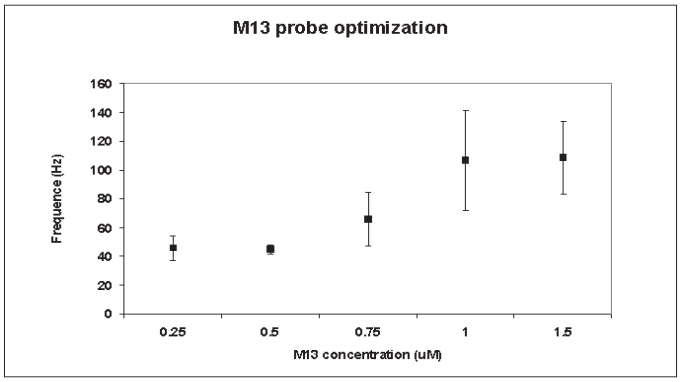

c.

Figure 1 **a)** Resonant Frequency Change by Avidin Immobilization after MPA Monolayer Forming on Gold Surface of Quartz Crystal. **b)** Result of avidin immobilization. **c)** Result of detection probe immobilization.

Detection of Plasmid HPV DNA and Non-HPV infection Samples by QCM Sensor

PCR amplicon from 11 Plasmid HPV DNA samples represented by the specific band of each HPV as well as 35 DNA without HPV infection samples were used to verify our developed sensor. A 1:100 dilution of DNA were denatured by heating at 95 °C before performing the hybridization assay. The QCM sensor revealed a parallel result comparing to an agarose gel electrophoresis (Fig.2). Significant differences ($p<0.0001$) of the detection signal was found between positive and negative control samples. Negative Cut off value for QCM detection was also determined from 35 samples with negative HR-HPV. Negative cut off value of 43.01 was estimated from mean +3SD (20.06 Hz +/- 7.46).

Sensitivity of QCM sensor

Plasmid HPV type 45 DNA from different concentration of serially diluted HPV type 45 (Dilution 100 to 10^7 copy/μl) was extracted using the protocol described before. Regular PCR was carried out using DNA extract from each concentration of cells. The gel electrophoresis detection of PCR products is shown in Fig.3a. On the gel, from left to right are the DNA size marker, PCR product for cell concentration of 10^7 copy / μl , regular PCR products for cell concentrations from 10^0 to 10^7 copy/μl , and the PCR blank control (purified water was used as non-template control). Electrophoresis confirmed the successful amplification of the correct size of PCR products (190 bp). Using electrophoresis, the detection limit for regular PCR products of HPV type 45 is approximately 10^3 copy/μl. PCR products for HPV type 45 (10^0 to 10^7copy/μl) were tested by QCM sensor. The frequency shifts resulted from the detection is shown in Fig. 3b. The frequency change of the sensor was enhanced as the plasmid concentration of the PCR products increased. The negative control (PCR products using purified water as PCR template) was also tested. The threshold for the positive detection is set as negative signal + 3 SD (negative cut off = 43.01 Hz) and the detection limit was determined as 10^3 copy/μl. (= 44.7 Hz)

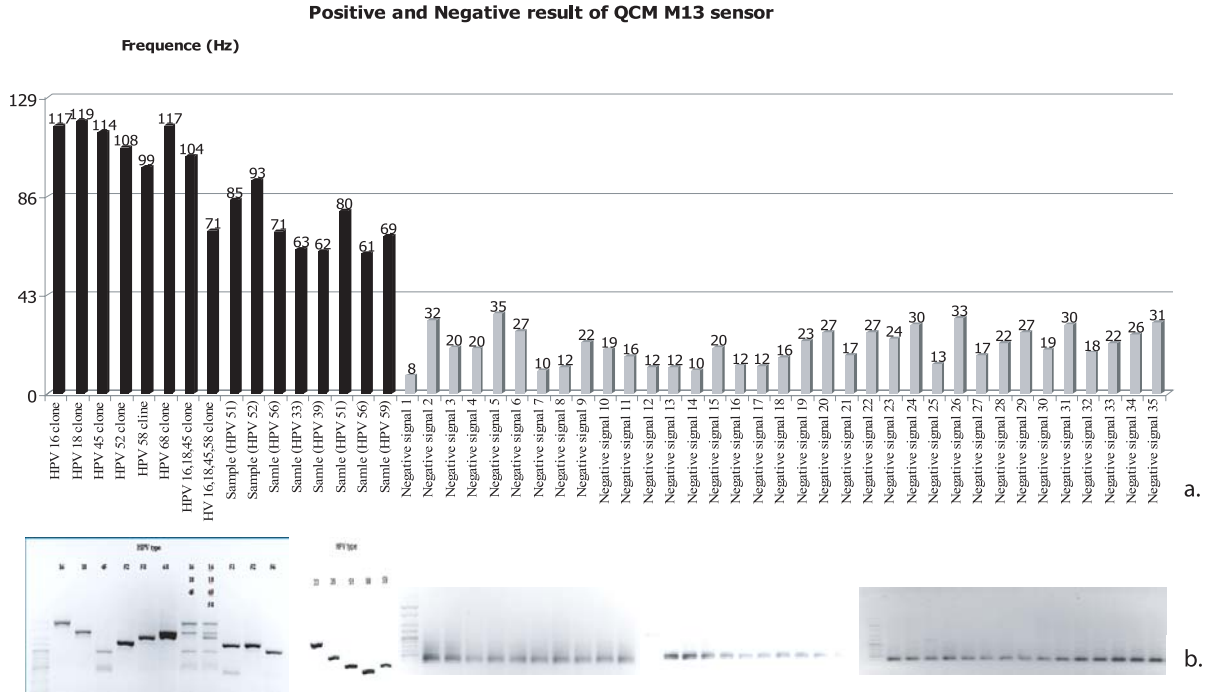

Figure 2 Frequency and PCR amplicon result of QCM sensor. a).illustrated the frequency changes from PCR amplicon of 11 plasmids HPV DNA and 35 non-HPV infectious samples. b) PCR amplicon from 11 Plasmid HPV DNA samples presented the specific band of specific type of each HPV and 35 HPV non-infection samples

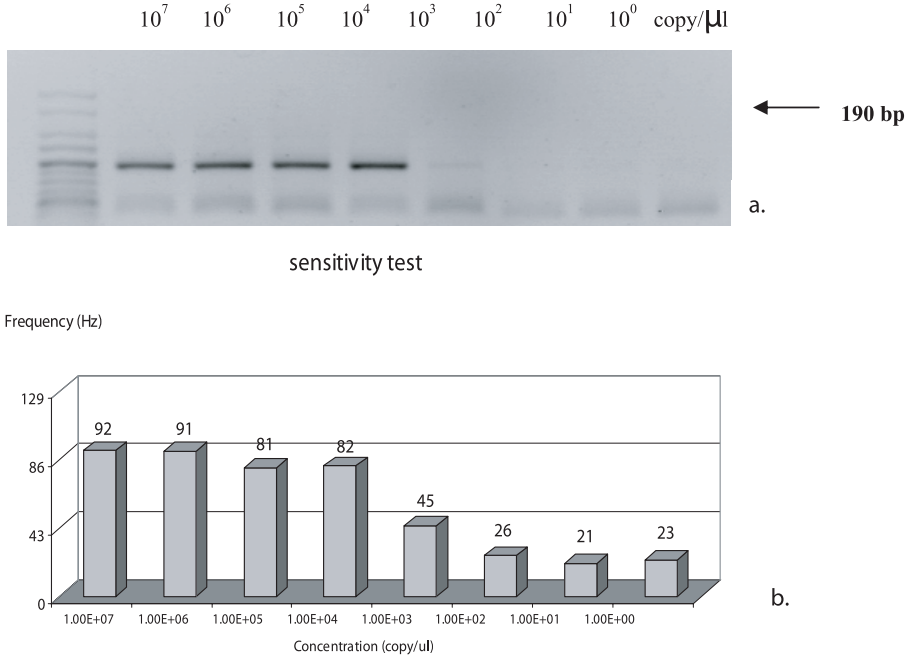

Figure3 a) Gel electrophoresis detection of HPV type 45 PCR products. PCR product for plasmid concentration of 10^7 copy/μl; 7?0, regular PCR products for cell concentrations from 10^7 to10^0 copy/μl

b) The responses of the QCM M13 sensor to different PCR samples. The detection limit is determined as 10^3 copy/μl.

Precision of QCM sensor

Precision was evaluated using a protocol based on hybridization between QCM sensor and PCR product encoded detection probe sequence procedure. PCR products of HPV type 45 DNA plasmid as template were used to estimate precision. Target values for positive signal were > 43.01 Hz (above negative cut off). Only one sample, aliquot to 12 numbers, were analyzed in four time a day for 3 days. Between-run precision was calculated from the results obtained during these 3 days. (Result Mean = 87.83 Hz, SD=15.95, CV%= 18.16). Within-run precision was estimated by the four time difference value for used in the calculated optimal precision. (Result Mean = 86.86 Hz, SD = 5.9, CV% = 6.85). The frequency shifts resulted from the precision study is shown in Fig. 4

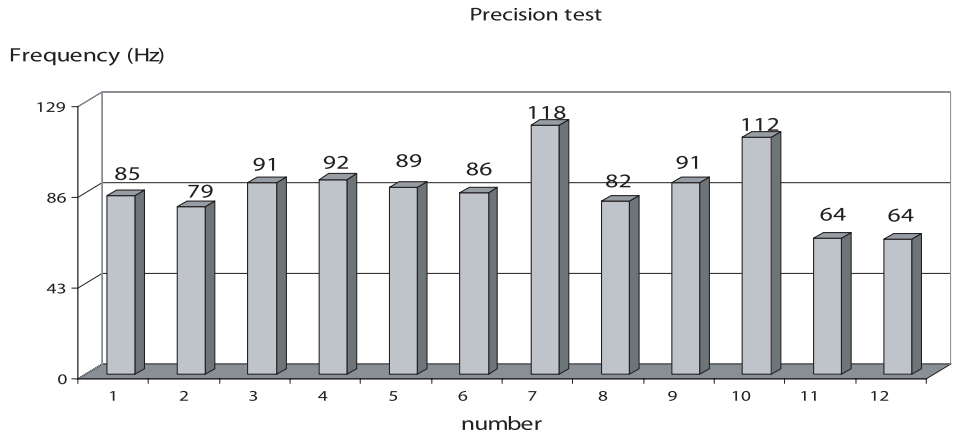

Figure 4 The frequency shifts resulted from the precision study in four time a day for 3 days.(Number 1-4 for day 1, Number 5-8 for day 2 and Number 9-12 for day 3)

Discussion

Identification of HR-HPV is clinical relevance to use as a surveillance marker for risk assessment of cervical cancer development. DNA sensor was selected on the basis of the successful achievement of DNA hybridization for the rapid detection of high-risk HPV type. There are two major issues that must be dealt with in order to produce a sensitive and precision QCM based DNA biosensor for detection of the target of interest: the strategy used for oligonucleotides detection probe immobilization on the quartz crystal and the optimization of experimental conditions to minimize non-specific hybridization. In this study, the 5'-biotinylated oligonucleotides detection probe immobilization was attached on avidin via MPA formed self assembly monolayer to the gold surface. Immobilization not only helps in forming the required close proximity between the biomaterial and the transducer, but also helps in stabilizing it for reused (D'Souza 2001). This immobilization technique is widely reported to be one of the most efficient and simple immobilization methods available (Zhou, Huang et al. 2001;

Tombelli, Mascini et al. 2002). The main advantage of this method is that non-specific amplification can be decreased since only a perfect hybridization between the templates and the oligonucleotide probe.

The reliability of QCM based DNA biosensors developed in this study was verified using positive and negative HR-HPV. Sensitivity of detection is 103 copy/μl in comparable to PCR detection on agarose gel electrophoresis. In addition, the QCM sensor reveals that non-HR-HPV samples giving response frequency shift less than 43.01 Hz (negative cut off).

The disadvantage of the QCM system versus the gel electrophoresis post PCR detection is concerned to several washing and drying steps which require about 4 hours for preparation process. However, Tombelli *et al.* (Tombelli S 2000; Tombelli, Mascini *et al.* 2002) and Caruso *et al.* (Frank Caruso 1997) had reported the coated crystal in the similar system. Generally, it could be kept at 4°C for several weeks without losing their activity.

Using of liquid phase flow system instead of gaseous phase measurement was suggested as an interesting solution to solve this drawback of QCM. However, the detection of high-risk HPV by using this specific M13 sensor provides many advantages including the label free DNA hybridization reaction (no toxic compounds are required, i.e. ethidium bromide)(MEA 1991),

Conclusion

QCM sensor was developed as a rapid detection of high-risk HPV type within 20 minutes. The optimal concentrations of MPA, avidin and 5'-biotinylated DNA probe for immobilization of specific DNA probe on gold surface of quartz crystal were found at 10 mM, 0.1 mg/ml and 1 μM, respectively. The QCM sensor was verified with 11 HR-HPV positive DNA as well as 35 non HR-HPV. This work provides the possibility of using quartz crystal microbalance systems as a biosensor to detect other specific DNA target amplification via additional oligonucleotides detection sequence.

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