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Advancements in electrochemical DNA sensor for detection of human papilloma virus - A review

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ABSTRACT

Human papillomavirus (HPV) is one of the most common sexually transmitted disease, transmitted through intimate skin contact or mucosal membrane. The HPV virus consists of a double-stranded circular DNA and the role of HPV virus in cervical cancer has been studied extensively. Thus it is critical to develop rapid identification method for early detection of the virus. A portable biosensing device could give rapid and reliable results for the identification and quantitative determination of the virus. The fabrication of electrochemical biosensors is one of the current techniques utilized to achieve this aim. In such electrochemical biosensors, a single-strand DNA is immobilized onto an electrically conducting surface and the changes in electrical parameters due to the hybridization on the electrode surface are measured. This review covers the recent developments in electrochemical DNA biosensors for the detection of HPV virus. Due to the several advantages of electrochemical DNA biosensors, their applications have witnessed an increased interest and research focus nowadays.

Introduction

One of the most common sexually transmitted infections is the human papillomavirus (HPV) which affects millions of people worldwide. Thus it has gathered huge attention due to increased focus on vaccine development and cancer-screening recommendations for early prevention of the disease [1]. While some common dermatological and sexually transmitted diseases are easily attributed to the presence of the HPV virus, there is still no cure for HPV [2], though a healthy immune system could offer sufficient protection from the virus itself. Around half of the world's population is exposed to the risk of HPV infection at least once in their lifetime [3]. Cervical cancer, from the contamination of high-risk HPV, is the third most prevalent type of cancer in occurrence and fourth in death rate among women worldwide [4,5]. The HPV-16 and HPV-18 are two of the most dangerous cancer-causing HPVs which contain the E6 and E7 oncogenes, are responsible for almost 70% of all cervical cancers. The HPV-16 is basically associated with squamous cell carcinoma, while the HPV-18 is associated with adenocarcinoma, a less common disease despite being more dreadful than the latter. Both the HPV 16 and HPV 18 are preventable by vaccination [2].

Around 26,900 new cases of HPV-associated cancers are diagnosed each year between 2004 and 2008, with 4100 women deaths from cervical cancer in the United States alone [6]. The National Program of Cancer Registries (NPCR) and the Surveillance, Epidemiology and End Results (SEER) program showed that an average of 33,369 diagnosed HPV-associated cancers are detected annually, including 12,080 males (8.1 per 100,000) and 21,290 females (13.2 per 100,000). The HPV virus is believed to be responsible for 96% of cervical cancers, 93% of anal cancers, 64% of vaginal cancers, 51% of vulvar cancer, 36% of penile cancers and 63% of oropharyngeal carcinomas [7].

The visible epidermal manifestations such as warts or condylomas, occur only in 1% of the infected patients. Therefore, the diagnosis of HPV infection requires specialized equipment to pinpoint the internal lesions at the mucous membranes. The Papanicolaou test is another type of analysis for proven cervical injury cases. In this test, the cervical

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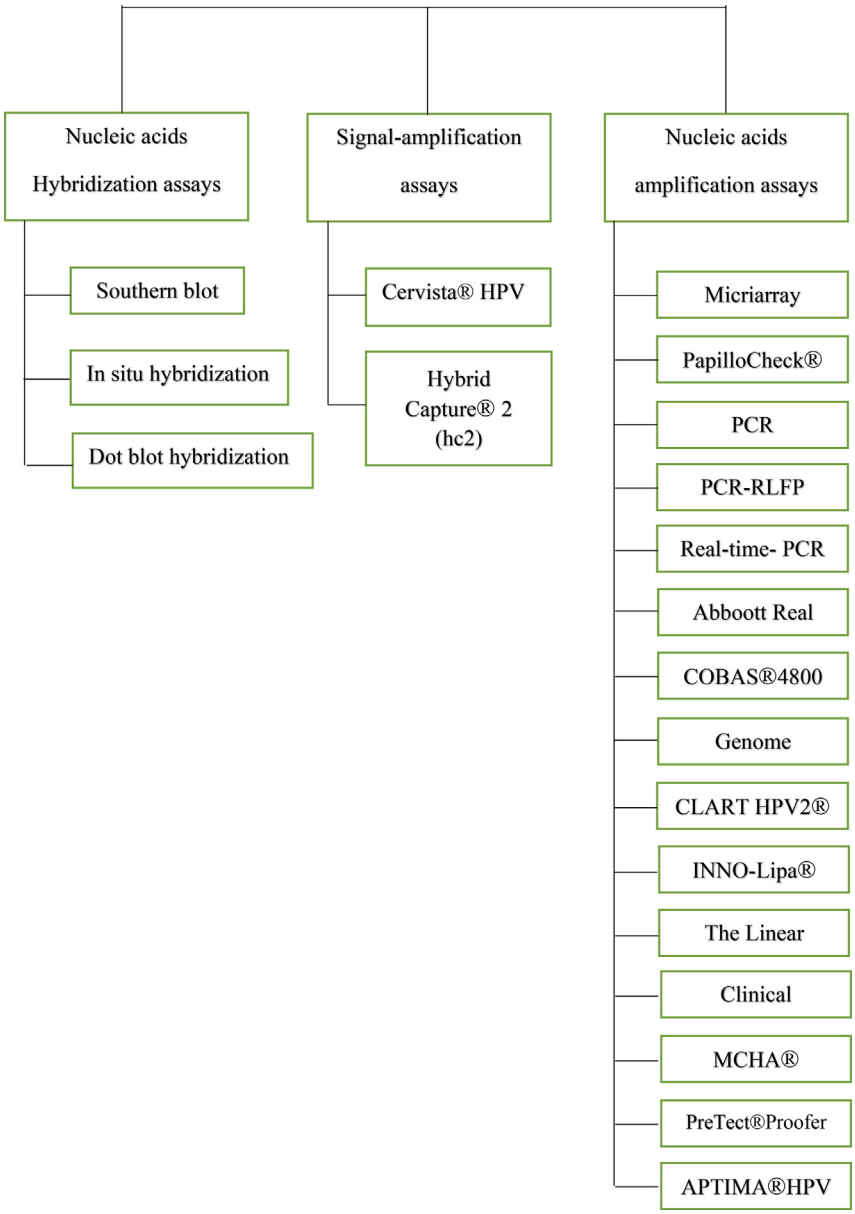
Table 1
Classification of molecular-biology techniques for HPV diagnosis.



cells are collected using swabs, followed by staining with the Papanicolau staining technique [8,9]. Molecular diagnosis is essential for the accurate differentiation of various HPV strains, which are categorized according to their low, intermediate or high oncogenic risk.

The differentiation of various HPV strains is a challenging task since all HPV strains are closely related. The protein expression ratio analysis could provide some differentiation of the various HPV strains through molecular fingerprinting of the oncogenic potential in histopathological samples [10,11]. The DNA analysis is another essential method of identification of the different types of HPV strains, which is based on the complementarity principle of the DNA strands. For example, the signal amplification of a fragment of target nucleic material can be identified by the polymerase chain reaction (PCR) method and signal amplification of an oligonucleotide hybridization assay [12,13]. At present, HPV diagnosis is based on molecular biological techniques which are categorized into nucleic acid-hybridization assays, signal amplification assays and nucleic-acid amplification (Table 1 and Table 2) [14].

Southern blotting, in situ hybridization, and dot-blot hybridization used radiolabeled nucleic acid hybridization assays to detect HPV infection in cervical samples. Low sensitivity, large amounts of purified DNA and time-consuming procedures are disadvantages of these techniques. However, generated high-quality information is benefit of using these techniques [14]. Southern blotting, in situ hybridization, and dot-blot hybridization used radiolabeled nucleic acid hybridization assays to detect HPV infection in cervical samples. Low sensitivity, large amounts of purified DNA and time-consuming procedures are disadvantages of these techniques. However, generated high-quality information is benefit of using these techniques [15]. The Hybrid Capture® 2 (hc2) distinguishes between high-risk and low-risk groups, but was not designed for genotyping single HPV [16]. The Cervista® HPV identifies the presence of 14 HR-HPV types and also utilizes a signal-amplification method for the detection of specific nucleic acids. The Cervista® assay demonstrated 100% sensitivity in the detection of CIN III and 98% sensitivity in the detection of CIN II compared to hc2 [17]. Lower false-positive rate, and high sensitivity and specificity to genotyping HPV -16/18 are other properties of this assay [18,19]. However, these techniques involve complex protocols and require specialized

Table 2
Advantages and Disadvantages of the HPV detection molecular methods.

Methods	Advantages	Disadvantages
Nucleic acids hybridization assays	• Southern blot is gold standard for HPV genomic analysis. • Presence of HPV in association with morphology.	• Low sensitivity, time consuming, relatively large. • Southern blot and hybridization cannot use degraded DNA • Purified DNA amount
Signal amplification Assays	• Quantitative • FDA-approved test (hc2) • Lower false-positive rate • High sensitivity to genotyping	• Licensed and patented technologies • Cannot use for genotyping individual
Nucleic acids amplification assays	• Flexible technology • Very high sensitivity • Multiplex analysis	• Lower amplification signals of some HPV genotypes • Contamination with previously amplified material can lead to false positives

skills for correct instrument operation because these techniques do not fulfill the requirements of point-of-care clinical diagnostics and generally lacks the required sensitivity and rapidity [20].

The early diagnosis of the disease is highly desirable, due to the dangers posed by the presence of HPV in the human body, thus more efficient techniques for the detection of HPV virus are needed [21]. The most widely used techniques for the screening and diagnosis of HPV infections are the Digene hybrid capture assay (HC2), pap-smear test and polymerase chain reaction with generic primers [22,23]. However, the first two techniques have some disadvantages. These methods involve the use of expensive instrumentation and expert analysis, yet display low specificity and sensitivity, hence are time consuming and complicated. Therefore, these techniques are unsuitable with limited resources and personnel [24]. Recently, some new detection techniques such as leaky surface acoustic wave, piezoelectricity and fluorescence spectroscopy, have been utilized for the detection of HPV virus, but these are also still expensive and require complicated instrumentation [25–27]. In this review, we cover some recent approaches and advances for the diagnosis and electrochemical DNA detection of HPV. We also compare their detection performance based on the sensor platform, sensing method, technique, detection limits and detection ranges.

Biosensors

Biosensors are integrated receptor-transducer devices which integrate a biological component with a detector component that has three main parts [28]:

(1) A biologically derived material as the sensing element.
(2) A transducer or detector element which is responsible for the conversion of biochemical signal from the recognition element into a readable and quantified output.
(3) A signal processor that displays the transformed signal.

In 1962, Clark and Lyons reported their innovative work on glucose sensor for the detection of glucose in samples from diabetic patients [29]. Currently the research interest towards biosensors is growing steadily due to their advantages such as sensitivity, specificity, rapid response and simplicity without prolonged experimentation processes and purification requirements.

Biosensors are commonly classified into two categories: i) the bioreceptor (whole cells, antibodies, peptides, nucleic acids and aptamers) and ii) the transducer; and in some cases, the bioreceptor is coupled with the transducer [30]. The different types of transduction based biosensors are electrochemical, optical, mass and electrical biosensors.

DNA biosensors

The sensitive element in nucleic acid-based biosensors generally comprises of a single-stranded DNA (ssDNA) molecule which allows very specific binding of nucleic-acid sequences on a surface before the hybridization with the complementary target nucleic acid [31,32]. There is a direct proportionality between the amount of target and the level of hybridization with the signal intensity which influences the sensor response [33]. The synthesis of DNA sequences with functionalized end groups is commonly reported, furthermore DNA purification from biological or clinical samples is easier compared to protein, as the former are more stable molecules. The rapid and reversible hybridization of ssDNA (probe DNA) and target nucleic acid in electrochemical DNA biosensors allows the regeneration of the sensor which offers rapid detection, simplicity, low cost and stability. The detection of DNA sequences represents a promising strategy for the early screening and detection, as well as the early diagnosis of diseases [34,35]. Due to the deficient screening options and exceedingly high fatality rates in developing countries, the fabrication of rapid and economical detection devices is profoundly critical. Numerous methods such as radiochemical, enzymatic, fluorescent, surface plasmon resonance spectroscopy and quartz crystal microbalance have been utilized for DNA detection [36–38]. In addition, it is also crucial to develop ultrasensitive devices for samples with lower concentration of HPV DNA. Several ultrasensitive sequence-specific DNA detection techniques such as polymerase chain reaction [39], rolling circle amplification (RCA) [40–42] and nucleic acid sequence-based amplification [43] were described, but most of these techniques involve complex operation, expensive reagents, dedicated instrumentation and exhaustive labels [44]. In addition, biosensors commonly involve the use of a wide range of transduction mechanisms such as optical, electrochemical, electrical and mass based mechanism.

Electrochemical DNA biosensors

The focus on electrochemical DNA biosensors has been increasing recently as they are low cost, portable, less complicated, offer rapid detection with higher sensitivity via signal amplification and are compatible with mass production with the help of microfabrication technologies. In addition, other methods of detection involves expensive signal transduction tools compared to the electrochemical detection of analytes which is the result of direct electronic signals, mainly the current (Amps) [21]. Moreover few electronic instruments could record very low currents down to several pico-Amps which improve the sensitivity of detection. Electrochemical DNA biosensors are based on the hybridization of target nucleic acid with their complementary DNA probes or ssDNA [45,46]. Over the recent years, numerous innovative advancements have been achieved with improved biorecognition and interfacial procedures on solid state devices and in solution. Biosensing provides some extraordinary prospects for genetic screening and recognition with the capability to produce features on solid substrates with nanoscale accuracy. Lately, a number of innovative improvements have been achieved in electrochemical DNA biosensors. The crucial role of the sensor is to facilitate the formation of probe target complex, where the coupling process generates a detectable signal into an

electronic readout.

In DNA biosensors, the single strand DNA (ss-DNA) molecules or the probe DNAs are the biological recognition element. This general concept is built upon the hybridization of ss-DNA probe with the complementary target DNA. The hybridization leads to changes in the mass transport, light emission or absorption, or proton concentration which generates the signal. The generated signal is transformed into a quantifiable response through a suitable transduction element such as thermal, optical or electrochemical, which converts the signal into current, light or potential [47].

An electrochemical DNA biosensor, consists of the working, reference and counter electrodes similar to a conventional three-electrode electrochemical cell [21,48]. The immobilization of the ss-DNA or DNA-analog probe onto the electrode surface is the first principle of an electrochemical DNA biosensor. The hybridization of ss-DNA probe with the target DNA induces a variation in the electrochemical signal of the electroactive labels/indicators linked to the probes [49–52]. Electrodes such as gold, hanging mercury drop electrode, platinum and various types of carbon electrodes modified with the probe ss-DNA molecules are the most common working electrodes (WE), while a saturated calomel electrode (SCE) and platinum wire are the reference and counter electrodes, respectively [53]. Electrochemical techniques are well suited for DNA diagnostics because the direct recognition is achieved through techniques such as voltammetry, potentiometry, amperometry and electrochemical impedance spectroscopy (EIS). Therefore, there is no requirement for advanced and expensive signal transduction equipment. In addition, the immobilized probe sequences could be established on different electrode substrates and detection could be performed with an electrochemical analyzer [54].

Electrochemical DNA sensors for HPV detection

The electrochemical recognition of HPV-DNA sequences has been developed recently [55–57]. The increased sensitivity, small sample volume, low cost, simplicity and portability make the electrochemical detection an excellent candidate for point-of-care DNA diagnostics. Although the electrochemical detection of DNA is already an established method with increasing number of publications annually, most of the reports suffer from the lack of analyzing real human samples, and their assessment with standard approaches [58]. Fig. 1 shows DNA detection of both synthetic oligonucleotides (HPV16E7p and HPV45E6) was performed using a so-called sandwich type format [55].

Direct (label-free) electrochemical DNA detection

A label-free electrochemical DNA detection is a direct method of measuring the physiochemical variations on the surface of transduction device due to the DNA hybridization. The electrical transduction could be combined with advances in nanotechnology which gives rise to electrochemical DNA nanosensors, as it is portable and more economical compared to the label-free physical and optical transduction [59]. This method excludes the requirement for an additional marker (label) step which allows more simple sensing protocol [53,60–62]. This section is in chronological order.

In 2008, Liepold et al. introduced a new electronic microarray format (EDDA) that could display hybridization of amplicons in a few minutes [63]. Dipstick-type microelectrode arrays consisting of 32 gold microelectrodes on silicon, was applied directly to the amplification reaction without any labeling or washing steps. Initially, the gold microelectrodes were functionalized with the probe DNA and followed by the hybridization of ferrocene-modified DNA. Immobilization procedure, accessibility of the capture probes, and prevention from non-specific target adsorption are a prerequisite for a robust and reliable performance of the sensor and these aspects are demonstrated in this research. The minimal equipment needed in this proposed sensor and make it suited for clinical and medical diagnosis tests and it is easy to handle. Labeling or washing steps are not needed in this work. The target DNA was hybridized with the probe DNA as the sensor was dipped into the sample. With this approach, the target DNA could be measured as a decrease in the electrochemical signal. The HPV-6 target DNA could be measured down to 30 pM accuracy using this method.

In another investigation, Zari et al. determined a 20-mer oligonucleotide of HPV virus adsorbed on screen-printed gold electrodes (SPGE) for the direct monitoring of DNA hybridization with the absence of labeling or an external redox indicator [64]. The electrochemical oxidation of purines (guanine and adenine) and DNA fragments at various electrode surfaces (carbon paste, glassy carbon electrode and gold) were investigated by square wave voltammetry (SWV). The guanine and adenine bases could be quantitatively determined by SWV for a deoxyribonucleic acid sample after the acid hydrolysis. The gold electrodes showed the highest sensitivity. The order of peak currents was found to increase as follows: hybrid-modified SPGE < 11-base mismatched modified SPGE < 18-base mismatched SPGE < probe modified SPGE. The selectivity of the proposed DNA biosensor was examined by the non-complementary oligonucleotides. Under optimal conditions, this sensor shows a good calibration range with a HPV DNA sequence detection limit of 2 pg ml-1 (S/N = 3). The proposed protocol is very simple and relatively rapid. The main advantage of this method is the suitability of microarray fabrication, as electrochemical microarrays are easily fabricated using gold electrode, while the carbon or mercury based electrodes are unsuitable.

For the first time, Piro et al. revealed a technology based on the application of a conjugated copolymer poly (5-hydroxy-1,4-naphthoquinone-co-5-hydroxy-2-carboxyethyl-1,4-naphthoquinone) for the detection of HPV infection [65]. The proposed immunosensor acts as the immobilization and transduction element and is able to identify the interaction between the antigenic peptide L1 from the HPV-16 major capsid protein, a dominant epitope involved in viral infection, as well as

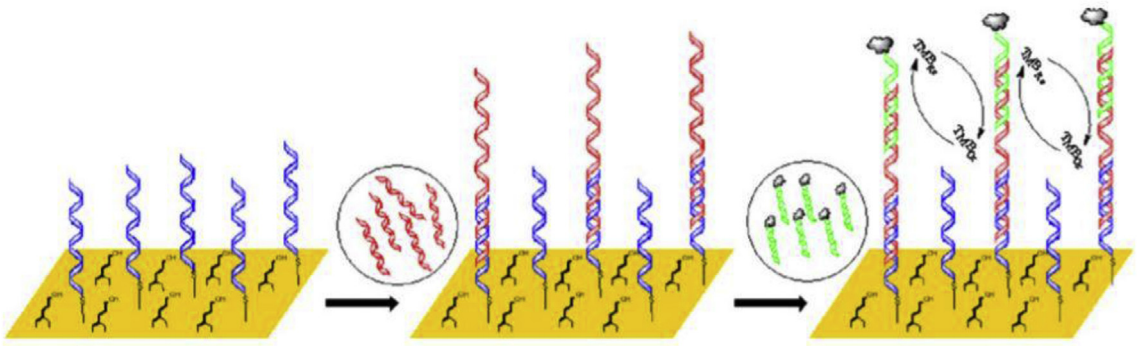


Fig. 1. Schematic description of the developed assay based on co-immobilization of thiolated probe with backfiller, hybridization process and electrochemical detection.

in prophylactic vaccine and the relevant antibody. The HPV-16-L1 was grafted as the probe to detect the HPV-16 antibody. The SWV of the poly(HNQ-co-HNQ-COOH) recorded in PBS before and after the grafting process, as well as after the complexation with αOVA and αHPV. The anti-OVA induces a weaker signal drop compared to the anti-HPV (available in serum). The significant advantage of this technique is that the antigen–antibody complex formation takes place without any intermediate steps or reagents by monitoring the electrochemical response of the polymer by SWV. Moreover, the interest to replace Ag probe (in this case, HPV virus) by much smaller fragments such as an oligopeptide of a few tens amino acids, which presents much smaller size than a bulky antigen was studied. By this approach the system in terms of sensitivity and stability might improve. This report was based on the use of synthetic DNA.

In a similar work, the analytical performance of polyaniline-multiwalled carbon nanotube film (PANi–MWCNT) on interdigitated platinum electrode arrays (IDA) for the detection of HPV virus was reported by Dai Tran et al. [66]. They reported a sensitivity of $1.75 \pm 0.2\,\mu A\,nM^{-1}$ ($r^2 = 0.997$) between 10 and 50 nM of anti-HPV concentration with a limit of detection (LOD) of 490 pM. This work highlights the ability of the arrays to control the technical and biological variation. However, further studies are required to determine the viability of the assays for clinical applications.

Wang et al. fabricated an ultrasensitive biosensor by electrochemical deposition of gold nanoparticles on single walled carbon nanotube (SWCNTs) arrays for the detection of human hepatitis B and papilloma viruses [67]. They examined both the aligned and random SWCNTs arrays upon the immobilization of ss-DNA probe onto the SWCNTs/Au surface by a self-assembly method [68]. The experimentally achieved detection limits are almost the same for the random and the aligned SWCNTs/Au arrays, the electrochemical detection of HPV virus was performed by electrochemical impedance spectroscopy (EIS). The hybridization detection of a 24-base papilloma virus ss-DNA showed great stability where the charge transfer resistance dropped to less than 1% after a month of storage between 4 and 8 °C, with a very low detection limit of 1 amol. In this research horizontally aligned SWCNTs array coated with Au NPs is a very promising platform for DNA detection but making parallel SWCNTs array represents a huge challenge in practice, it requires a very steady and stable laminar gas flow and it is easier to fabric the random SWCNTs array. The remarkable improvement of SWCNTs/Au platforms over those utilizing SWCNTs or Au NPs alone indicates synergistic interactions of SWCNTs array and Au NPs. Moreover, good regeneration ability of the proposed biosensor in hot water makes it a valuable candidate for achieving early diagnosis of gene-related diseases. The considerable advantage of this work is the very low limit of detection (LOD) compared to the previous report. This report was also based on the use of synthetic DNA.

In another report, Huang et al. constructed an enzyme-free and label-free electrochemical biosensor based on graphene/Au nanorod/polythionine (G/Au NR/PT) modified glassy carbon electrode (GCE) for the ultrasensitive detection for HPV DNA. A ruthenium complex, $[Ru(phen)_3]^{2+}$ was selected as the redox indicator [69]. The proposed DNA biosensor demonstrated great selectivity and a LOD of 1.0×10^{-13} to 1.0×10^{-10} M and 4.03×10^{-14} M, respectively, for the detection of HPV DNA. The presence of graphene enhances the electrode surface area and electrical conductivity. The enhanced immobilization of the probe DNA and the ability for hybridization is due to the presence of Au NRs. The proposed biosensor showed very low detection limit and the experiment was optimized by different factors, and the target DNA (TD) was also detected in complex human serum sample. The fabricated biosensor shows good potential for clinical applications. Campos-Ferreira et al. proposed a label-free electrochemical DNA biosensor for the identification of a target gene cloned into a plasmid [70]. A 23-mer guanine-free oligonucleotide was immobilized on pencil graphite electrode for the detection of E6 gene (due to its clinical importance) from HPV type 16 virus. The LOD is 16 pg/μL and suggests that the electrochemical method is more sensitive and specific compared to the agarose gel electrophoresis assay (1500-fold lower in concentration) which can be postulated as a new and alternative method for cloning analysis in plasmids. The DNA biosensors are less commonly explored for cloning analysis and the considerable advantage of this novel, simple, inexpensive, stable electrochemical DNA biosensor is the ability to detect the HPV 16 E6 gene cloned in the expression vector.

Karimizefreh et al. reported a label-free electrochemical impedance biosensor for the recognition of HPV type 16 [71]. Instead of using a common gold disk electrode, they utilized a modified glassy carbon/gold nanosheet electrode to improve the performance of the DNA biosensor because among different morphologies of gold nanostructures, gold nanosheets have many edges and corners and can serve as more active sites for catalysis compared to spherical nanoparticles. This electrode was immobilized with thiolated single-stranded 25-mer-oligonucleotide (synthetic thiol-ss-DNA) and gave a LOD of 0.15 pM. Moreover, the biosensor showed one base pair mismatch detection limit with good selectivity. The biosensor has a great potential in HPV DNA diagnostics and clinical analysis.

Kowalczyk et al. for the first time, investigated the role of mercury (II) in the nitrogen base for the voltammetric detection of specific DNA sequences characteristic of the HPV type 18 in thymine bases without the labeling step [72]. The detection of target DNA sequence was based on the electrochemical reduction of mercury (II) ions bonded with opposite thymine bases (mispairs) in the DNA helix. The laser ablation technique coupled with inductively coupled plasma mass spectrometry and circular dichroism was also utilized in this detection. This fabricated biosensor is convenient, low cost, less construction procedure and showed very low LOD and quantification limit of $(1.2 \pm 0.2) \cdot 10^{-14}$ and $(7.5 \pm 0.1) \cdot 10^{-14}$ M, respectively, and have a great potential in HPV DNA diagnostics. Limited success has been achieved for the commercialization of the above biosensors.

Indirect (labeled) electrochemical DNA detection

Although the label-free DNA detection allows an easier screening step and decreases the time consumption and expenses, it does not achieve the sensitivity of the labeled DNA detection. The indirect technique improves the sensitivity to attomolar concentrations of the target DNA [73]. The indirect techniques also need mediators to simplify the electron transfer step between them and the electrode. The most common electron mediators are $Fe[(CN)]_6^{3-/4-}$, $Os(bpy)_3^{3+/2+}$ $Ru(bpy)_3^{3+/2+}$, ferrocene and methylene blue (MB). Nanomaterials such as metal nanoparticles could also be utilized as indirect electrochemical sensors, which represent a large redox reservoir [74].

In 2008, Sabzi et al. utilized MB as an electroactive label on a pencil graphite electrode (PGE) to provide a well-defined recognition interface for the detection of HPV target DNA [57]. Five non-complementary DNAs corresponding to the human, HCV, fungi and bacterial genomes were investigated to confirm the selectivity of the biosensor towards HPV DNA detection in the presence of other DNAs. The data showed that the fabricated electrode detects the target DNA with a LOD of 1.2 ng/μl. The authors attempted to discriminate the HPV universal region from the mixed oligousenucleo HCV universal, bacterial 16S rDNA and fungi 5/18s rDNA regions by using DNA biosensors in the future work. In a similar study, MB was utilized as an electrochemical intercalator to monitor the DNA hybridization process [75]. The MB binds specifically to guanine bases in the ssDNA and a lower current signal was observed upon the DNA hybridization. Souza et al. fabricated an electrochemical DNA biosensor for specific sequence detection of the E1 HPV type 16, using MB as the hybridization indicator utilizing the DPV technique. To examine the selectivity of the biosensor, some hybridization experiments with non-complementary oligonucleotides (target and probe DNAs were the synthetic oligonucleotides) were performed. They reported that the DNA biosensor could be utilized for the detection of E1 HPV gene due to enhanced selectivity of the DPV

technique. The LOD of the probe immobilized on the electrode to its complementary sequence was 1.49 nM.

In another fabricated biosensor, L-cysteine was first electrodeposited on gold surface to form the L-cysteine film and followed by the immobilization of HPV16-specific probe on the modified surface [46]. The DPV technique was used to investigate the reduction signals of MB. The effect of probe concentration was analyzed and the best performance was obtained at 1000 nM. The biosensor demonstrated great sensitivity and comprehensive linear response to the target concentration (18.75 nM and 250 nM) with a LOD of 18.13 nM. The biosensor performance indicated a high sensitivity and selectivity towards HPV-16 detection and distinguished it from other types of HPV. This allows the possibility for developing new portable detection systems for different types of HPV viruses and could be the first step for effective diagnosis during the early stages of infection.

Hematoxylin was utilized as the biological stain in biomedical research and diagnostic procedures [76], as well as a pivotal agent in the histopathology of prostate cancer [77]. Nasirzadeh et al. studied the electrochemical properties of hematoxylin modified electrodes as an electrochemical sensor for the detection of NADH [78], hydrazine [79] and other biological compounds [80] in their previous works. In another report, Nasirzadeh et al. introduced a new electrochemical biosensor based on the interaction of hematoxylin with 20-mer deoxyoligonucleotides of HPV through hybridization [81]. In this research, the best method for the self-assembly of DNA probes is the drop-casting self-assembly method while the best hybridization is the solution-based hybridization method. The electrochemical techniques utilized in the detection were cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The electrical signal under optimum conditions shows a linear relationship with the concentration of target DNA, ranging from 12.5 nM to 350.0 nM with a LOD of 3.8 nM. Although the miniaturization and economic cost are the key elements for an ideal biosensor, the simultaneous detection of multiple analytes is also much desired. For this reason, Civit et al. developed an electrochemical genosensor array for the simultaneous detection of three specific high-risk HPV sequences, HPV-16, 18 and 45 [56]. In this work, the parallel detection of multiple targets and multiplex studies were made possible by 16 gold WE sensor arrays, through the immobilization of three thiolated HPV probes on alternating electrodes. This work highlights the high specificity of the sensor arrays and no significant cross-hybridization between the three high-risk sequences was observed. Moreover, real samples obtained from cervical scrapes were amplified and were utilized to investigate the genosensor performance in real clinical applications, where an excellent correlation was obtained compared to the response of HPV genotyping carried out in a hospital laboratory.

Jampasa et al. designed a selective electrochemical biosensor for the detection of HPV type 16 DNA, based on the immobilization of anthraquinone-labeled pyrrolidinyl peptide nucleic acid (acpcPNA) probe [82]. The probe was immobilized onto a chitosan-modified disposable screen-printed carbon electrode via a C-terminal lysine residue using glutaraldehyde as the cross-linking agent. They obtained a linear range between 0.02 and 12.0 μM, with a LOD and limit of quantification (LOQ) of 4 and 14 nM, respectively. The fabricated biosensor showed high selectivity against non-complementary 14-base oligonucleotides, such as the HPV types 18, 31 and 33 DNA. The ease of electrode preparation and probe immobilization with small sample volumes are the main advantages of this platform. The excellent specificity under non-stringent hybridization conditions and simple instrumental set-up are some advantages of this technique for HPV screening in the developing countries. On the other hand, the requirement of DNA probe immobilization, the non-reusability and detection limits (LOD) higher than the standard DNA detection are some disadvantages of the proposed biosensors. Nevertheless, the detection limit is still acceptable to facilitate the detection of HPV type 16 DNA from PCR samples.

In 2015, Bartolome et al. modified glassy carbon electrodes (GC) with stable dispersion of pristine carbon nano-onion (CNOs) which were functionalized by diazonium salts possessing carboxylic acid or maleimide groups, using the electrografting technique [83]. They described two sensing platforms: i). GC/CNO/phenylacetic acid (PAA) immobilized with streptavidin and biotinylated capture DNA sequence and ii). GC/CNO/phenylmaleimide (PM) immobilized with thiolated DNA probe. Both the fabricated biosensors (sandwich type assay with a peroxidase labeled DNA reporter probe) were utilized for the HPV DNA sequence detection using the amperometry technique. The results distinctly showed better sensitivity and lower LOD of the amperometric assays achieved on the GC/CNO-modified surface compared to the bare GC electrodes. The proposed electrochemical DNA biosensor gave a sensitivity and LOD of 0.91 $\mu A\,nM^{-1}$ and 0.54 nM for the GCE/CNO/PAA (in the absence of CNO, sensitivity = 0.21 $\mu A\,nM^{-1}$; LOD = 3.9 nM) and 0.41 $\mu A\,nM^{-1}$ and 0.50 nM for the GCE/CNO/PM (in absence of CNO, sensitivity = 0.11 $\mu A\,nM^{-1}$; LOD = 1.4 nM), respectively. The results showed that the incorporation of CNOs on the surface resulted in higher sensitivities and lower detection limits, due to the enhanced electron transfer process at the CNO-modified electrodes. These observations confirm the promising and versatile role of the CNO-modified surfaces for the development of different and effective analytical sensor systems.

Bartosik et al. developed an electrochemical-chip based assay using an antidigoxigenin-peroxidase detection system on carbon screen-printed electrode chips [84]. They used a magnetic bead-modified DNA probe to capture the target HPV DNA. The designed HPV DNA biosensor showed high sensitivity in the attomolar range. This biosensor also demonstrated the selective discrimination of HPV-16 from HPV-18 which is highly significant for HPV-16-1961 and HPV-18-2031 targets in their relevant genomes, where around 75% of their sequences possess resemblance. Since the majority of assays still lack validation using real human samples ideally from patients, along with the comparison with standard methods, one of the most significant aspect of this assay is that the proposed biosensor could be an effective tool for the detection of HPV DNA directly from cervical samples. The assay was successfully utilized for the detection of DNA not only from cancer cell lines, but also from human cervical brush smears from HPV patients.

Very recently, Teengam et al. developed a novel paper-based electrochemical DNA biosensor for the determination of high-risk HPV type 16 using pyrrolidinyl peptide nucleic acid (AQ-PNA) probe immobilized on a graphene-polyaniline (G-PANI) modified electrode [85]. The AQ-PNA immobilization and target HPV-16 DNA hybridization were confirmed by EIS and SWV techniques. The biosensor gave a LOD of 2.3 nM with a linear range between 10 and 200 nM. The designed biosensor effectively detected the PCR amplified DNA from HPV-16 positive SiHa cells. The proposed biosensors showed some improved features compared to previous works. The electrochemical paper-based analytical devices (ePAD) provide a low-cost, disposable biosensor for point-of-care (POC) applications. Moreover, the G-PANI modified electrode is attractive relative to the covalent immobilization because of its inherent simplicity. Another advantage of this sensor is the possibility for mass production while limiting the variation among individual sensors, which is crucial for a disposable biosensor. Table 3 summarizes the electrochemical DNA biosensors for HPV detection. As can be seen, the suggested electrochemical sensor platforms have a low LOD and a wide linear range, compared to other types of DNA biosensors.

Conclusion and future prospects

Significant challenges remain for the development of powerful biosensors for the prevention of cervical cancer in the developing world. The medical diagnosis needs great advances that are widely dependent on the development of new materials and technologies for the fabrication of state-of-the-art biosensors. A low cost, user friendly, point-of-care diagnostic that can be used for self-sampling or clinic-based sampling of cervical cells is needed to simultaneously facilitate the screen-and-treat procedure in clinical diagnostics. The lack of

Table 3
Comparison of electrochemical biosensor for HPV Diagnostics.

Electrode	Electroactive Indicator	Electrochemical Technique	Linear range	Detection limit (LOD) (nM)	Ref.
L-cysteine film/Gold electrode	Methylene blue	DPV	18.75–250 nM	18.13	[46]
Oligonucleotide/Gold electrodes	Horseradish peroxidase	Steps & Sweeps	0.1–10 nM	490 pM	[55]
Gold electrodes	Horseradish peroxidase	Steps & Sweeps	0.1–10 nM 0.1–12 nM	220 pM 170 pM	[56]
Pencil Graphite Electrode	Methylene blue	SWV	0–10 ng/μL	1.2 ng/μL	[57]
Carboxyphenyl layer/GCE	Mercury(II)	SWV	10^{-15}-10^{-6} M	$1.2{\cdot}10^{-5}$	[62]
Dipstick-type microelectrode gold arrays	Ferrocenium	ACV&CV		30 pM	[63]
Screen-printed gold electrode	Free-indicator	SWV	0–5 ng ml^{-1}	2 pg ml^{-1}	[64]
Poly(HNQ-co-HNQ-COOH) film/GCE	Free-indicator	SWV	50 nM		[65]
Interdigitated platinum electrode arrays/Polyaniline/Multiwalled Carbon Nanotube film	Free-indicator	CV & SWV	10–50 nM	490 pM	[66]
Single walled carbon nanotube arrays/Au nanoparticles/SiO_2/Si Substrate	Free-indicator	EIS	10^{-9} - 10^{-3} nM	10^{-9}	[67]
Graphene/Aunanorod/Polythionine/GCE	$([Ru(phen)_3]^{2+}$)	DPV	10^{-4}-10^{-1} nM	$4.03{*}10^{-5}$	[69]
Pencil Graphite Electrode	Free-indicator	DPV	40–5000 pg/μL	16 pg/μL	[70]
Glassy carbon electrode/Gold nanosheets	Free-indicator	EIS	1 pM-1 μM	0.15 pM	[71]
Graphite electrode	Methylene blue	DPV	2–10 nM	1.49	[75]
Gold electrode	Hematoxylin	CV & DPV	12.5–350 nM	3.8	[81]
Screen-printed carbon/Chitosan	Anthraquinone	SWV	0.02–12 μM	4.0	[82]
Glassy Carbon/Carbon nano-onion	Horseradish peroxidase	Amperometry	0–20 nM	0.54	[83]
Graphene/Polyaniline	Pyrrolidinyl	EIS & SWV	10–200 nM	2.3	[85]

routine home testing is another drawback of the current detection systems due to their off-line sample preparation and reagent handling. The unique features such as high specificity, mutual recognition between short oligonucleotides and a complex long-sized eukaryotic genome and high physicochemical stability, could make DNA biosensors as excellent candidates for nano-system fabrication. The ability to distinguish different strains of the same organism, especially when isolated from different geographical locations is one the great advantages of DNA biosensors, as well as the high chemical stability compared to enzymes and antibodies. However, the presence of interfering substances in complex real samples is a major challenge because the current biosensors are performed with pure, synthetic modelled-DNA sequences. The detection approaches described in this review have their own merits, but those based on indirect electrochemical DNA biosensors are the most interesting due to their enhanced sensitivity down to the femto molar range with possible miniaturization. Future HPV biosensors still require improvements in the current technology with a user friendly interface without compromising the accuracy and reliability. There is still a long road towards commercialization and clinical realization and biosensors as high-throughput devices require huge investments for mass production and commercialization which could be serious obstacles for small start-up companies. However, the distinctive advantages of nucleic acid biosensor technologies could transform the healthcare sector in developing countries.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ab.2018.07.002.

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