INVITED PAPER

Advances in Nanotechnology and Microfluidics for Human Papillomavirus Diagnostics

This paper presents the existing assays and platforms employed for Human Papillomavirus (HPV) diagnosis, and highlights recent advances in nanotechnology and microfluidics that potentially enable new approaches for HPV diagnosis.

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ABSTRACT | Human papillomavirus (HPV) has been shown in many studies as a prerequisite for the development of cervical cancer, which is the second most common and predominant form of malignancy among women worldwide, with 270 000 deaths (80% of whom live in developing countries) and 500 000 new cases per year. Early diagnosis of cervical cancer allows patients to be fully treated. Therefore, there is high clinical utility of HPV diagnostics even with binary positive/negative indication of the presence of HPV in patient samples. Although the Pap smear is widely used for cervical cancer screening, this method still suffers from low sensitivity and specificity. Thus, simple, rapid and inexpensive diagnostic methods are needed to detect the etiology of cervical cancer, i.e., HPV, especially high-risk oncogenic subtypes 16 and 18. Here, we review the existing assays and platforms employed for HPV diagnosis, and highlight recent advances in nanotechnology and microfluidics that potentially enable new approaches for HPV diagnosis.

KEYWORDS | HPV; microfluidic; nanotechnology; point-of-care diagnosis; sensors

I. INTRODUCTION

Human Papillomavirus (HPV) is the primary risk factor of cervical cancer, which accounts for 10%–15% of cancer-related deaths in women worldwide [1]–[4]. About 270 000 women per year, 80% of whom live in less developed and developing countries, die of cervical cancer [5]–[7]. For instance, the incidence rates range from less than 33.4 per 100 000 in Latin America up to 87.3 per 100 000 in South Eastern Africa (see Fig. 1) [6]–[8]. Cervical cancer is highly curable, if diagnosed in the early stages [9]. HPV can be an early indicator for cervical cancer, and HPV testing can provide a more reliable and sensitive indicator than traditional diagnosis methods [10]. Therefore, there is a significant clinical value of detecting the presence and degree of HPV infection in patients [8]. In developed countries, existing cervical cancer screening procedures with molecular probes have greatly decreased the burden linked to this disease [11]. However, in developing countries such as India, screening for cervical cancer is primarily performed by cytological testing [12], which has inadequate sensitivity, as well as poor specificity and reproducibility [8]. The Pap smear test (also called the Papanicolaou test, Pap test, or smear test) is highly subjective to the interpretation of the cytologist, and it shows considerable variation in sensitivity (17%–99%) and specificity (9%–100%) in many cases [13]. For instance, one-third of women who

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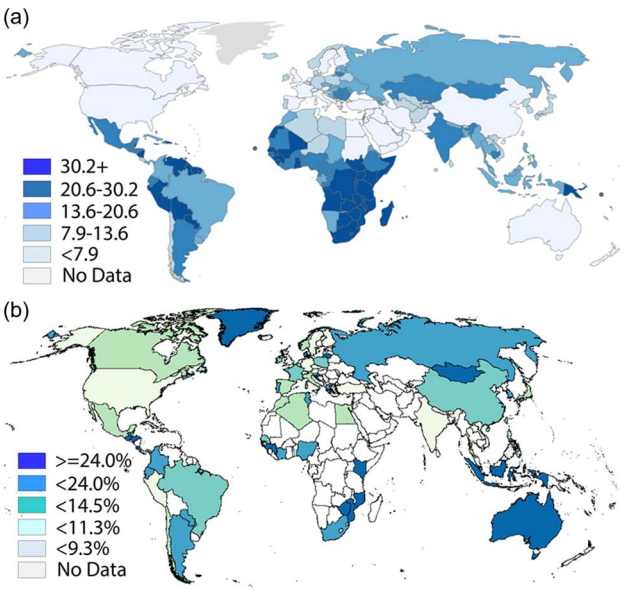

Fig. 1. *(a) Cervical cancer distribution around the world: Cervical cancer cases per 100 000 people based on data from the International Association of Cancer Registries, 2012. High incidence is observed in Africa and South America [21]. (b) Worldwide HPV prevalence: Estimate for HPV prevalence by country based on data compiled by the Institut Català d'Oncologia (ICO) Information Centre on HPV and Cancer, updated 2014 [22].*

have normal Pap smear test results progress to cervical cancer [12]. Additionally, cytology-based screenings require a laboratory infrastructure and skilled cytologists, which are not available in most of the less developed and developing countries, thus enabling only a small percentage of women in these regions to have active screening and timely treatment [6]. Due to large populations, lack of skilled technicians, and limited budgets in developing countries, there is limited coordination and enforcement of standard screening procedures [14]. Hence, there is a need to develop inexpensive, robust screening techniques to improve the quality of cervical cancer screening in developing and resource-limited countries [2], [15].

Cervical cancers gradually progress over a long period of time, which allows for cancer screening procedures to detect early neoplastic alterations. In the general progression of cervical cancer, there are three different detection peaks among women: 1) transient infections with carcinogenic types of HPV during their teens and 20s, 2) cervical precancerous conditions around their 30s, and 3) invasive cancers at 40 to 50 years of age [8]. Thus, the American Society for Colposcopy and Cervical Pathology (ASCCP) recommends HPV screening at peak ages of curable precancerous phases. Their recommendations include cytology every three years between the ages of 21 and 29, then cytology and HPV screening every five years (or continued cytology screening every three years) between the ages of 30 and 65 [10], [16]. The conventional prevention programs in developing countries rely on repeated series of cytologic examinations, including Pap smears, and colposcopy (visual examination of the cervix using a microscope to evaluate abnormal tissue and assist with directed biopsies for further pathological examination) [8], [17]. Alternatively, the HPV vaccines Cervarix and Gardasil prevent infection with high-risk HPV types 16 and 18, with Gardisil offering protection against types 6 and 11. The World Health Organization (WHO) and public health officials recommend vaccination of young women against HPV to prevent cervical cancer and to decrease the number of treatments for cervical cancer precursors.

In addition to being the primary risk factor for the development of cervical cancer, HPV has also been implicated in cancer at several other sites such as the oropharynx, vagina, anus, and penis [2], [15]. Therefore, novel diagnostic techniques could also be used to screen for these other cancers caused by HPV.

Further, HPV, the most common sexually transmitted virus, is comprised of more than 100 different genotypes [18], [19]. The mucosal types are divided into high-risk (HR) and low-risk HPV that have diverse biological origins and oncogenic potential. HPV-16/18 are the most well-known HR-HPV species that typically cause cervical cancers in women, and HPV-associated cervical diseases place a significant burden on the healthcare system [20]. In this review article, we 1) focus on the present HPV diagnostic technologies, 2) highlight the gap between present assays and clinical HPV diagnostic patient needs, and 3) evaluate the potential of nanotechnology and microfluidics to develop point-of-care (POC) diagnostics for HPV.

II. ADVANCES IN NANOTECHNOLOGY AND MICROFLUIDICS FOR HPV DIAGNOSTICS

Nanotechnology and microfluidics hold great promise to develop biosensing platforms for ultrasensitive detection of HPV. Table 1 summarizes these platforms, which have each been applied to HPV diagnostics. These biosensing platforms typically contain a detection area, where a HPV biomarker is sensed using optical-, electrical-, magnetic-, mass- or acoustic-based biosensing platforms. Most of these biosensors monitor HPV nucleic acids (i.e. DNA and RNA) in clinical samples for diagnostics. In these platforms, target amplification methods, such as polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP), are commonly used to produce millions of target HPV nucleic acid amplicons. The following nucleic acid hybridizations can be carried out within a detection area where specific HPV capture nucleic acid probes are immobilized. Moreover, antibody-antigen interactions have also been applied for HPV diagnostics, primarily by sensing infected cells.

Table 1 Comparison of Biosensor Technologies for HPV Diagnostics

Detection Method	Biomarker	LOD	Reference
Fluorescence	HPV-16, HPV-18, HPV-45 (Biotinylated DNA strands)	10^3 copies μL^{-1}	[23]
	Biotinylated HPV DNA strands	20 pM	[24]
	HPV-16 HPV-18 (DNA strands)	0.17 nM 0.78 nM	[25]
	HPV-16 HPV-18 (DNA strands)	70 pM 60 pM	[26]
	HPV DNA strands	1 fM	[28]
	Biotinylated HPV DNA strands	25 pM	[30]
	HPV-16 (DNA strands)	0.7 copies per cell	[32]
	HPV-6b (DNA strands)	< 33 copies μL^{-1}	[36]
	HPV-6, HPV-11, and HPV-16, HPV-18 (DNA strands)	10 copies μL^{-1} 102 copies μL^{-1}	[34]
	HPV 16 oligonucleotide	0.1 μM	[41]
	HPV 16 sequence SiHa cell line	10^{-6} μM 20 cells μL^{-1}	[44]
	On-chip nucleic acid extraction	5 cells μL^{-1}	[46]
	Molecular beacon-based oligonucleotide detection (HIV presented, HPV not given)	10 nM	[47]
Nanoparticle-based optical	HPV-6, HPV-11, HPV-16, HPV-18 (DNA strands)	50 nM	[51]
	HPV-16, HPV-18 (Biotinylated DNA strands)	30 pM	[52]
	HPV-16, HPV-18 (DNA strands)	0.14 nM	[53]
	HPV DNA strands	50 pM	[54]
Electrochemical	HPV-16 HPV-18 HPV-45 (DNA strands)	0.22 nM 0.17 nM 0.11 nM	[57]
	HPV-16 (DNA strands)	18 nM	[58]
	HPV DNA strands	3.8 nM	[59]
	HPV-16 (DNA strands)	4 nM	[60]
	HPV-6 (DNA strands)	30 pM	[61]
	HPV-16 (antibody)	0.49 nM	[63]
Magnetic	HPV-39 (Biotinylated DNA strands)	10 pM	[64]
Mass	HPV-58 (DNA strands)	100 copies (0.8 pg/mL)	[71]
Surface acoustic wave	HPV-18 (DNA strands)	1.21 fg/mL	[73]
	HPV-18 (DNA strands)	1 fg/mL	[74]

A. Optical Detection Technologies

Fluorescence-based detection: Fluorescence-based detection has been utilized to detect HPV nucleic acids using nano-engineered labels. For instance, organic and biofunctional nanocrystals were used as labels for quantitative detection of different HPV genotypes (HPV-16, HPV-18, HPV-45) in the range of 10^3–10^5 copies/μL [23]. These streptavidin-coated nano-sized crystals were

composed of labels including fluorescein diacetate encapsulated within polyethylene glycol (PEG)-Amine-modified phospholipids. They were incubated on a detection surface and immobilized with HPV DNA capture probes after target biotinylated HPV DNA hybridization. For fluorescent signals, an enzymatic reaction was employed by adding dimethyl sulfoxide (DMSO) and sodium hydroxide (NaOH) on the detection area. These nanocrystals increased the detection signal since they carried multiple fluorescent precursors. Fluorescent dye-doped streptavidin-coated sili, ca nanoparticles were also utilized as labels to detect biotinylated HPV DNA at concentrations as low as 20 pM [24]. In this assay, fluorescent signal intensity was measured directly without any enzymatic reaction.

Multiplexed detection of HPV-16 and HPV-18 was demonstrated in a sandwich assay format with a limit of detection (LOD) of 0.17 and 0.78 nM, respectively [25]. Avidin-coated silica nanoparticles were functionalized with biotinylated capture probe HPV DNA strands, which were then mixed with 64-base HPV-16 and HPV-18 target DNA strands. After hybridization, target DNA strands were selectively captured on the nanoparticles. Then, fluorescein amidite (FAM) and 6-carboxyl-X-rhodamine (Rox) labeled HPV-16 and HPV-18 detection probes were incubated with particles to identify target DNA strands. Magnetic microparticles (MMPs) were also used as capture surfaces for target HPV DNA strands [26]. MMPs functionalized with HPV-16 and HPV-18 capture probe DNA strands were simultaneously mixed with HPV-16 and HPV-18 target DNA and HPV-16 and HPV-18 detection probe DNA strands with the functionalized labels. These labels were quantum dots (QDs) coated with multiple FAM- or Rox-labeled random DNA for targeting HPV-16 and HPV-18, respectively. After hybridization, MMPs were magnetically separated from the sample followed by a heating step to release the labels inside the solutions. Afterwards, HPV-16 and HPV-18 were detected by simply measuring the FAM and Rox fluorescent signal at concentrations down to 70 and 60 pM, respectively.

Target HPV DNA was also detected using QDs. [27]. After the hybridization of target DNA with biotinylated capture probe DNA and QD labeled detection probe DNA, streptavidin-coated magnetic beads were added to the solution. DNA complexes were immobilized on the magnetic bead surfaces due to biotin-streptavidin binding. Afterwards, the beads were magnetically trapped in the solution and the supernatant was collected to sense remaining QD labeled detection probe DNA, so as to detect target DNA using fluorescent measurements, where fluorescent signal was inversely proportional to target DNA. Thus, HPV-16 DNA was qualitatively detected in 160 clinical cervical swab samples.

A polymer-based biochip was recently developed to distinguish between the 12 most common HPV genotypes [29]. 36 DNA probes were chosen and printed with polydimethylacrylamide (PDMAA) on a substrate in a microarray pattern via a UV-irradiation process. Isolated and amplified DNA was then stained with streptavidin-Cy5 and hybridized to the area of the microarray with corresponding genotype-specific capture probes. In this way, fluorescent readings from the chip indicate the genotype of an HPV sample down to 10^4 copies with an overall LOD of 10 copies.

HPV DNA was also analyzed in a microfluidic channel using microbeads as capture surfaces for target HPV DNA (see Fig. 2) [28]. First, microbeads were confined to chambers in a microfluidic channel. These beads were then functionalized with the capture probes. After target HPV DNA was introduced inside the channel and hybridized on the beads, horseradish peroxidase (HRP)-functionalized gold nanoparticles with secondary HPV DNA probes were incubated with the target. The HRP enzymatic reaction was then initiated by adding hydrogen peroxide (H_2O_2) with biotin-tyramine. This reaction resulted in biotin deposition on the bead surfaces coupled with the oxidation of biotin-tyramine. Streptavidin-labeled QDs were subsequently incubated inside the channels to bind deposited biotins, and fluorescent signal was measured to quantify target HPV DNA down to 1 fM. This method had a LOD of 10 copies/μL of HPV genomic DNA.

In another study, a single layer array of microbeads functionalized with specific HPV DNA capture probes was used as a capture surface in a microfluidic platform [30]. A mixture of different spectrally encoded microbeads was utilized for multiplex detection of HPV-6, HPV-11, HPV-16, and HPV-18 target DNA. After the hybridization of biotinylated target DNA with probe DNA in the platform, streptavidin-phycoerythrin (i.e., fluorescent label) was introduced in a microchannel. The generated fluorescent signal was then measured on the beads. With this method, HPV DNA can be detected down to 25 pM.

Using the same microbead technique, a set of 46 probes was developed and patented by Luminex Corporation that will hybridize to a wide range of HPV genotypes [31]. Each probe may be hybridized to a different colored microsphere, and a spectrophotomer or a flow cytometer can then be used to evaluate the subset of beads that are fluorescent and therefore the types of HPV present in the sample.

HPV-16 target DNA was also sensed in a capillary channel using a single molecule imaging system [32]. Target HPV DNA was hybridized with a fluorescent labeled HPV DNA probe and the resultant fluorescent signal was detected down to 0.7 copy per infected cell. Fluorescence resonance energy transfer (FRET) was applied by staining hybridized DNA with YOYO-3 dye, used as an acceptor to further increase the selectivity of the previous assay. Another single molecule detection system carried out quantitative detection of HPV-16 DNA hybridized to probes confined to a glass surface using both a single-probe and a dual-probe strategy [33]. In single probe mode, DNA was fluorescently stained prior to hybridization, achieving a LOD of 0.7 copy/cell. In the dual probe mode, a DNA strand was used as a fluorescent probe, effectively reducing background signal and increasing the detection

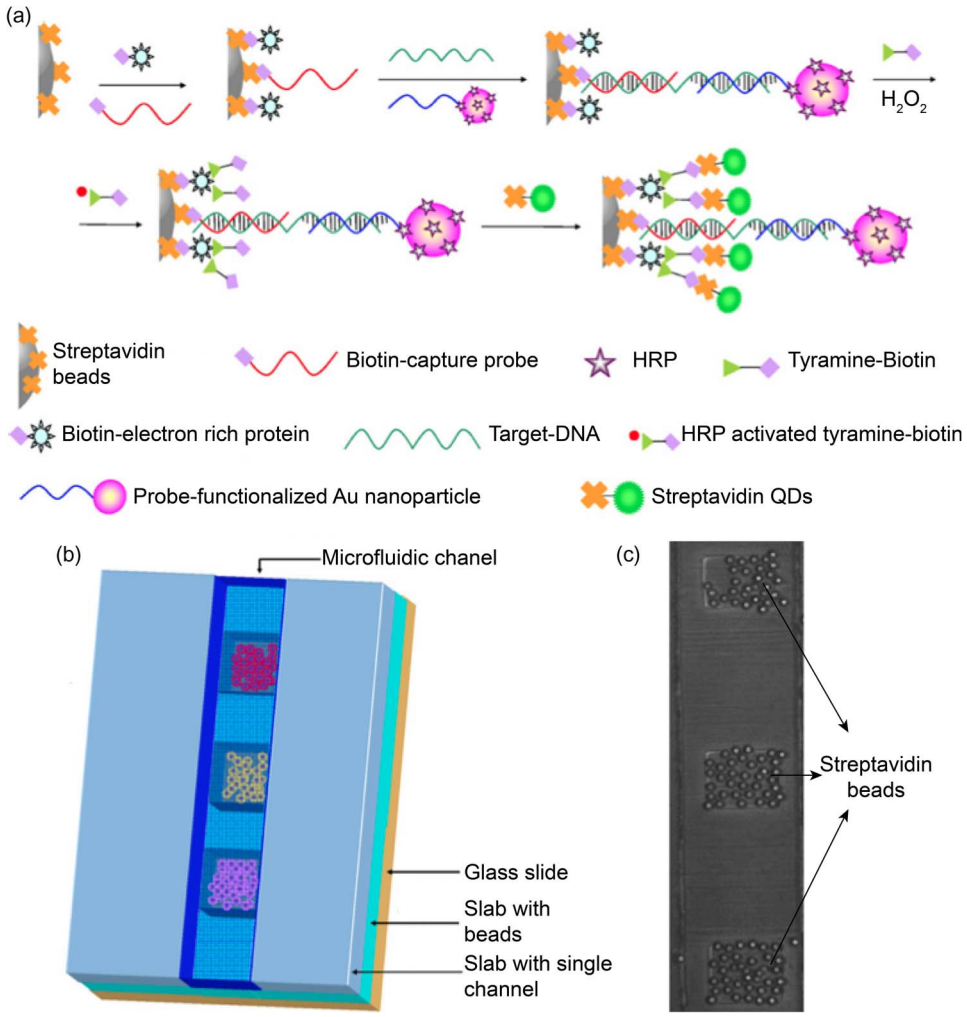

Fig. 2. ***HPV-DNA analysis in a microfluidic device. (a) Illustration of HPV-DNA detection protocol. Streptavidin coated beads grafted on microfluidic channel surfaces were functionalized with biotinylated capture DNA and biotin-electron rich protein. Then, target DNA and HRP labeled Au nanoparticles with detection DNA were hybridized on the magnetic bead surfaces. By introducing H_2O_2 with biotin-tyramine, the enzymatic reaction of HRP on Au nanoparticles was initiated, resulting in deposition of biotin molecules on the magnetic bead surfaces. Then, streptavidin labeled QDs were incubated to detect deposited biotin content, which was proportional to the target DNA concentration, via fluorescent measurements. (b) Schematic view and (c) micrograph of microfluidic channel covered with beads for HPV-DNA analysis. Reproduced with permission [28].***

range to five orders of magnitude, which is a clinically significant range, which covers early infection to carcinogenic stages.

A lateral flow DNA biosensor was developed to simultaneously detect 13 HPV genotypes (see Fig. 3) [34]. In this sensor, a nitrocellulose membrane contained an array of DNA capture probes specific to target HPV DNA and fluorescent-labeled DNA probes. The sample migrated on the array by capillary action and the fluorescent signal on the specific array area indicated the presence of target DNA from a particular subtype. This disposable sensor allows rapid (less than 30 min) analysis of samples with LOD values of 10 copies/μL for HPV-6, HPV-11, and HPV-16, and 10^2 copies/μL for HPV-18.

Another lateral flow chip with an array of HPV DNA capture probes was used to detect biotinylated target HPV DNA using a fluorescence-streptavidin mechanism [35]. With this configuration, the chip showed selective detection of HPV-16 and HPV-18 within 30 min.

Amplification strategies with fluorescent detection: On-chip PCR strategies have been developed and applied to increase detection sensitivity of different HPV genotypes. For instance, a microfluidic chip with a single reaction channel was used to amplify and label target DNA after PCR amplification [36]. The resultant biotinylated PCR product was hybridized on the channel surface and immobilized with specific DNA probes; streptavidin-(R)-phycoerythrin was then introduced. Fluorescent signal

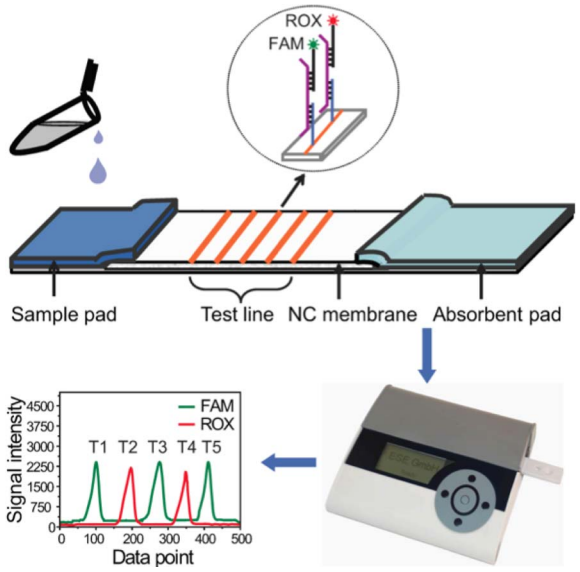

Fig. 3. *Illustration of a lateral flow based microfluidic assay for HPV-diagnostics. Fluorescence based detection was conducted to test different HPV genotypes using fluorescein amidite (FAM) and 6-carboxyl-X-rhodamine (Rox) labels on a nitrocellulose (NC) membrane. Reproduced with permission [34].*

intensity was measured to detect HPV-6b down to 100 copies per sample. This chip allowed multiplex detection of DNA targets using an array of DNA probes. In other studies, RT-PCR was performed on microfluidic chips for detection of HPV-16 mRNA using a minute sample volume (10–100 nL) [37], [38].

Real-time detection of HPV 16 was performed using NASBA, an isothermal method specifically designed for amplification of RNA [39], [40], on a microfluidic chip [41] (Fig. 4). The microfluidic chips were composed of 10- and 50-nL silicon-glass microchambers to detect 1.0 μM HPV at 41 °C. A thermal control unit and an optical detection platform were integrated into the setup. Yeast tRNA and Sigmacote were used to prevent adsorption of molecules (e.g., enzymes and targets) to microchamber walls. The microchips were designed to be disposable to prevent potential contamination. The results matched well with the results of conventional NASBA techniques, but with only 1/2000 of the sample size needed compared to the conventional technique [42]. This technology was recently applied to detection of E6 and E7 mRNA, which is closely associated with HPV, on a POC platform [43]. The device is capable of performing pre-concentration,

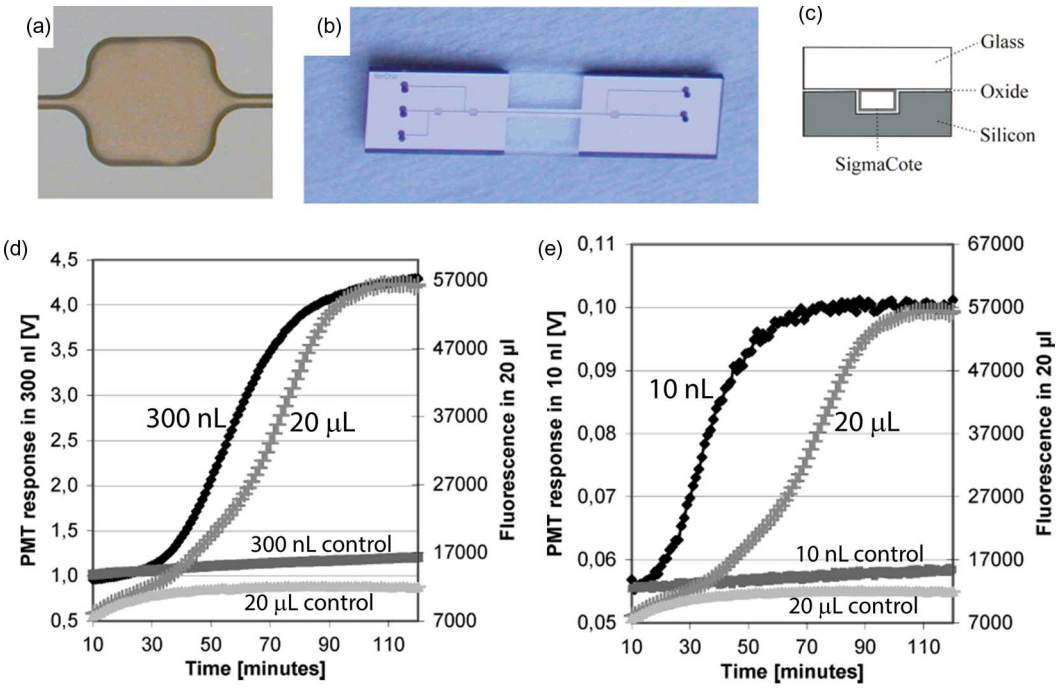

Fig. 4. *Real-time detection of HPV using microchips and NASBA. (a) An image of a 10-nL reaction chamber with dimensions of 450 × 450 × 50 μm³. (b) An image of the microchip, 5 × 20 mm. (c) Cross-sectional area composed of glass, oxide region, silicon, and SigmaCote. (d) Real-time NASBA of HPV-16 oligonucleotides performed in glass capillaries and in conventional polypropylene tubes for: (i) 1.0 μM HPV-16 in 300 nL, (ii) negative control in 300 nL, (iii) 1.0 μM HPV-16 in 20 μL, and (iv) negative control in 20 μL. (e) Real-time NASBA of HPV-16 oligonucleotides performed in a 10-nL reaction chamber and in conventional polypropylene tubes for: (i) 1.0 μM HPV-16 in 10 nL, (ii) negative control in 10 nL, (iii) 1.0 μM HPV-16 in 20 μL, and (iv) negative control in 20 μM. Reproduced with permission [41].*

extraction, amplification, and fluorescent detection, and requires minimal user interface. Future work on this platform aims to integrate the entire process onto a single chip for fully automated detection.

A real-time NASBA assay for detection of artificial HPV-16 sequences (the subtype that accounts for 54.4% of cervical cancer [15]) was evaluated [44]. An isothermal amplification technique was used in a multipurpose microfluidic technology platform made of cyclic olefin copolymer (COC). This technique used injection-molding to incorporate supply channels and ten parallel 80 nL COC microchannels to allow parallel channels to be filled at the same time as the NASBA mixture is added to a single inlet. A PEG coating was employed to avoid adsorption of molecules to COC microchannel walls. The mixture remained segregated during the process of reaction. The microfluidic device utilizes a custom-made optical detection unit, and is capable of real-time molecular beacon detection (Fig. 5). For HPV-16, the platform reached a sensitivity limit of 10^{-6} μM. Another disposable NASBA microchip detection platform was presented with enzymes, primers, and reagents stored in COC microchannels and re-hydrated by adding sample to initiate amplification [45].

Then, a portable, fully integrated platform was developed to perform: 1) chip-based total sample preparation, 2) automated extraction of nucleic acid from human cell samples, 3) amplification, and 4) fluorescent detection [see Fig. 6(a) and (b)] [46]. The samples were fixed in PreservCyt, a methanol based solution that can fixate epithelial cells at room temperature for at least one month. In this study, the major aim was extraction of HPV mRNA from cervical liquid based cytology specimens and on-chip detection. This platform used 3 mL of sample and achieved the extraction in a disposable COC chip of $64 \times 43 \times 3$ mm^3. The reagents in this study for elution, washing, and cell lysis were stored on-chip by using valve actuated reservoirs to enable hands-free analysis. The extraction was achieved in two filter phases: 1) cell pre-concentration and 2) nucleic acid capture. Results using cervical fluid-based cytology specimens confirmed the extraction of HPV-mRNA [see Fig. 6(c)].

Recently, a multiplexed detection of nucleic acids as disease markers was performed within discrete wells of a microfluidic chip using molecular beacons and total internal reflection fluorescence microscopy (TIRFM) [47] (see Fig. 7). Using a 4×4 array of 200 pL wells, HPV oligonucleotides as well as human immunodeficiency virus-1 (HIV-1), Hepatitis A (Hep A) and Hepatitis B (Hep B) oligonucleotides were screened. Target oligonucleotides were detected and distinguished against alternative oligonucleotides with different sequences. Results showed that for the HPV molecular beacon, only the matching HPV

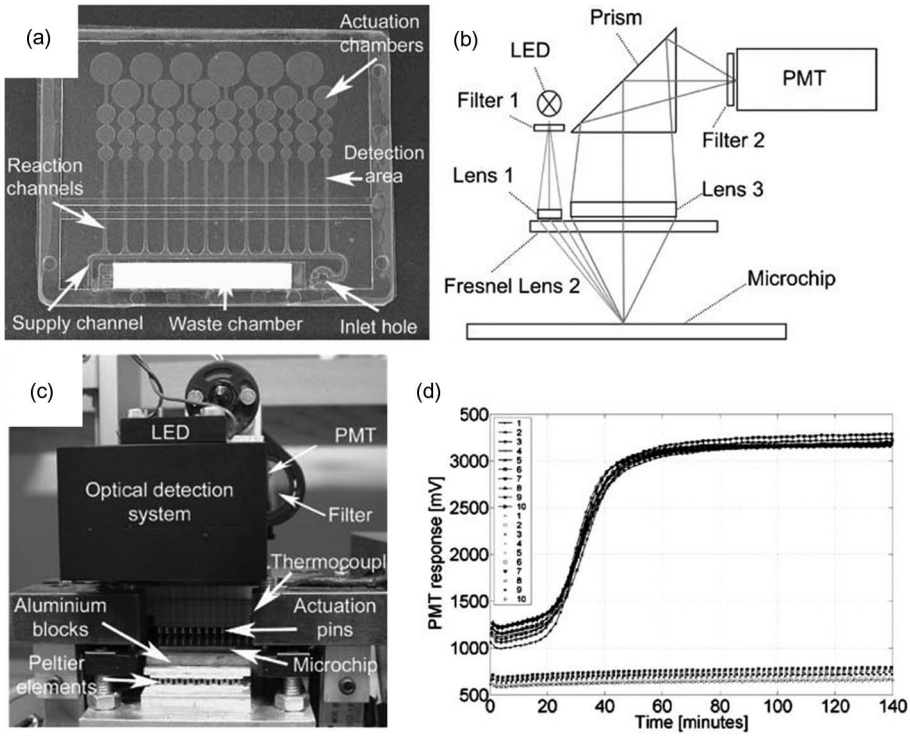

Fig. 5. ***Multiplexed real-time HPV detection using polymer microchips. (a) An image of the cyclic olefin copolymer (COC) microchip with a width of 50 mm and height of 40 mm. (b) Illustration of the custom optical setup. (c) An image of the components in the setup. (d) Real-time detection of 0.1 μM HPV-16 sequences on a microchip. Curves indicate positive amplification reactions. Negative controls give a low, nearly constant signal. Reproduced with permission [44].***

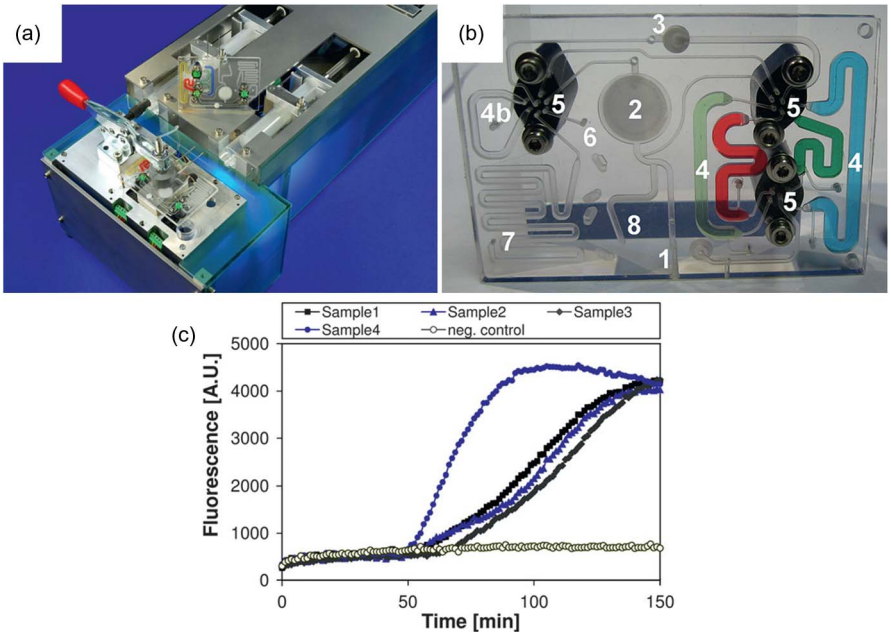

Fig. 6. ***Hands-free multiplexed real-time detection of HPV. (a) Image of the automated platform for chip-based sample preconcentration, nucleic acid extraction, amplification, and fluorescent detection. (b) Compartments corresponding to each step: (1) sample inlet, (2) cell filter, (3) solid phase extraction chamber, (4) reagent storage, (4b) storage DMSO/sorbitol, (5) turning valves, (6) waste outlet, (7) sample outlet, (8) pressure sensor. (c) During NASBA amplification, fluorescence intensity as a function of time is plotted. The filled markers (S-shape curves) correspond to amplification curves. The open circles (almost constant) represent the negative control. Reproduced with permission [46].***

target oligonucleotide showed a statistically significant positive signal. Currently, this platform requires a fluorescence detection system and TIRFM, which prevents portability for resource-constrained settings. However, at the expense of sensitivity, a less-advanced detection setup (e.g., a handheld fluorescence reader) can be integrated with the platform to enable portability for POC applications.

Nanoparticle-based optical detection: Nanoparticles and nanostructures have been previously utilized to detect and capture viral biomarkers and intact viruses without

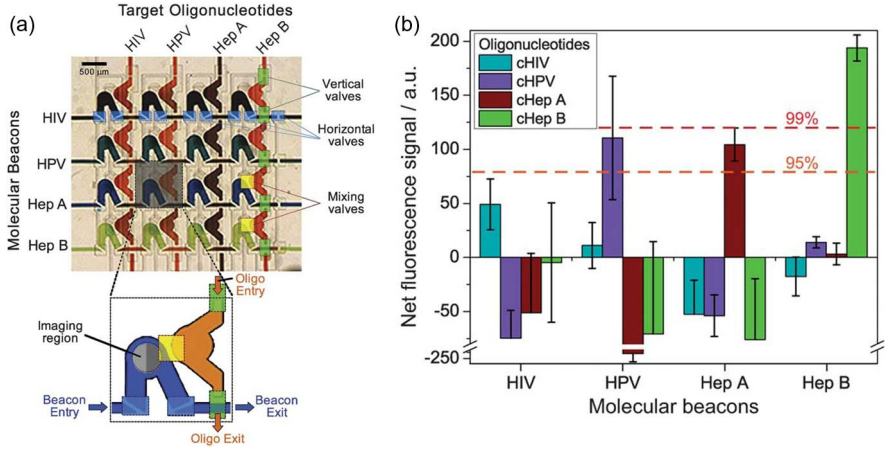

Fig. 7. ***Multiplexed detection of HPV in a combinatorial screening microchip. (a) An image of the microfluidic screening chip composed of 16 sets of two 200 pL sized half-wells. To show combinatorial capabilities, the chip was filled with dye solutions. Valves in the horizontal (blue boxes) and vertical (green boxes) directions were used to control fluid flow to the wells. Mixing between the adjacent half wells is shown as yellow boxes. The inset illustrates two half-wells and the valves. (b) Results indicate specificity in the fluorescence response of molecular beacon to the corresponding oligonucleotide for each oligonucleotide tested: HPV, human immunodeficiency virus-1 (HIV-1), Hepatitis A (Hep A) and Hepatitis B (Hep B). Reproduced with permission [47].***

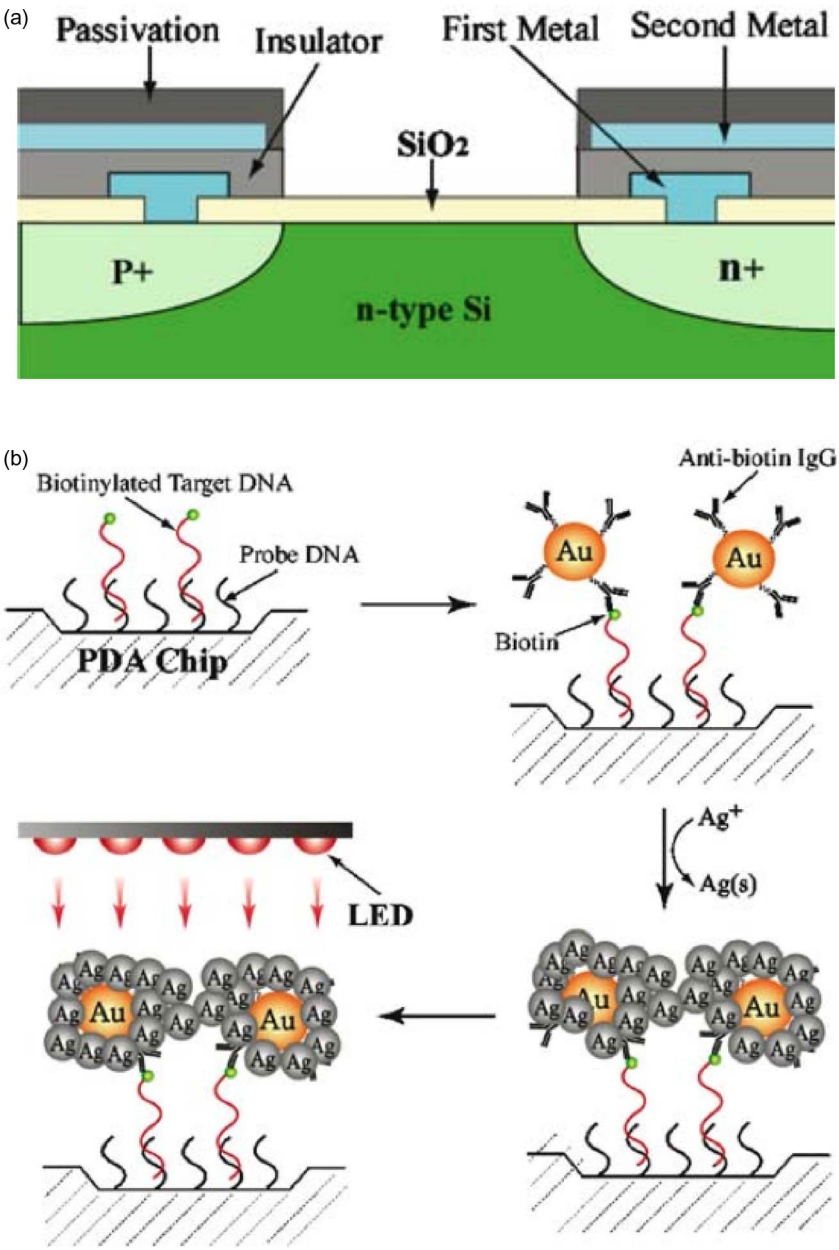

Fig. 8. ***Photodiode array (PDA) chip for nanoparticle-based detection. (a) Cross sectional view of a photodiode element of the chip. (b) Schematic illustration of the target DNA detection. Reproduced with permission [52].***

fluorescent-tags or labels [48]–[50]. An HPV genotyping DNA chip was developed to detect HPV-6, -11, -16, and -18 genotypes at concentrations down to 50 nM using gold/silver core-shell nanoparticle labels [51]. After HPV DNA capture probes were immobilized on a glass chip, target HPV DNA labeled with nanoparticles was captured on the glass surface via nucleic acid hybridization. Presence of HPV nucleic acid sequences was analyzed by measuring optical signal changes on the chip surface using a photodiode sensor.

Moreover, a bipolar integrated circuit photodiode array (PDA) chip was developed for DNA analysis, as shown in Fig. 8 [52]. The surface of the chip was functionalized with DNA probes to capture selectively biotinylated target DNA. As a label, anti-biotin antibody-conjugated gold nanoparticles were incubated on the hybridized DNA. Silver enhancement solution was then introduced on the surface to precipitate silver metal particles on the gold nanoparticles, which blocks the light on the PDA. Thus, target DNA was measured and quantified as a voltage drop on the PDA. Different HPV genotypes (e.g., HPV-16, HPV-18, HPV-56, and HPV-58) were analyzed on the chip at concentrations down to 30 pM.

A colorimetric detection method was also presented to detect HPV-16 and HPV-18 genotypes using sandwich hybridization of target HPV DNA between two HPV DNA probe functionalized gold nanoparticle layers [53]. After the hybridization, the salt concentration of the solution was increased. When no complementary sequences of DNA probes exist, gold nanoparticles aggregated and the solution color turned from red to purple. If target DNA was present between the gold nanoparticles, the solution maintained its red color. Facilitating this method, 70 mucous specimens from patients with cervical intraepithelial neoplasia were successfully tested with a LOD of 0.14 nM.

Another DNA chip was demonstrated to detect HPV-16 based on light scattering of silica nanoparticles, which were used as labels [54]. After sample DNA was hybridized with DNA capture probes and immobilized on the DNA chip, silica particles covered with sequences complementary to target DNA were incubated. The presence of target DNA was measured by light scattering from nanoparticles. With this format, a LOD of 100 pM was achieved. To improve the assay performance further, a dendrimer-like silica nanoparticle network was formed by hybridizing complementary DNA functionalized secondary silica nanoparticles on the primary nanoparticles. Using this strategy, target DNA was detected down to 50 pM. DNA could also be observed with the naked-eye down to 200 pM.

Ultrabright fluorescent mesoporous silica nanoparticles were also used to detect cancerous and precancerous cervical epithelial cells [55]. These particles were functionalized with folic acid to specifically target folate receptors of malignant cells. After 15 min incubation of the particles with the sample, HPV-16 infected cells were easily distinguishable from the normal cells. This method showed better sensitivity (95–97%) than HPV DNA and cell pathology tests (30–80%).

Other optical-based detection: Detection technologies based on antibody-antigen interactions have also been applied for HPV diagnostics. For example, a microfluidic device was developed to capture HPV-infected epithelial cells [56]. Expression of transmembrane protein R6 integrin is increased in HPV infected cells. Hence, the channel surface was functionalized with anti R6 integrin antibodies. After introducing the sample inside the channel, infected cells were captured on the surfaces, while other cells were washed away by applying adequate hydrodynamic forces. This method could offer quantitative measurement of HPV infection from a biopsy sample.

B. Electrical Detection Technologies

Electrochemical detection technologies have been employed to discern HPV genotypes. Gold electrode sensor arrays were developed for detection of HPV-16, HPV-18, and HPV-45 DNA sequences [57]. Detection was carried out in a sandwich assay format where target DNA was hybridized in between gold electrodes with thiolated capture probe DNA and horseradish peroxidase (HRP). Then, tetramethylbenzidine (TMB) was added on the sensor area, where HRP catalyzed the oxidation of TMB substrate, which was detected with electrodes using cyclic voltammetry. This biosensor allowed multiplex and quantitative detection of various HPV genotypes with electrode arrays where different thiolated DNA probes were functionalized. A LOD of 220, 170, and 110 pM, was reached for HPV-16, HPV-18, and HPV-45, respectively.

Another electrochemical biosensor was presented to analyze HPV-16 target DNA sequences down to 18 nM concentration [58]. To immobilize capture probe DNA, the surface of a sensor electrode was modified with an electrodeposited cysteine film. After hybridization of target DNA with the immobilized probe, methylene blue (MB) was introduced on the surface MB has a strong affinity to free guanine on the probe DNA and hybridization of target DNA with the probe causes the MB signal to decrease, allowing detection of target DNA. Measurements were conducted with a differential pulse voltammetry method to sense the reduced MB signal.

A similar approach was also conducted to measure HPV DNA with a LOD of 3.8 nM [59]. The gold electrode surface was covered with probe DNA and the hybridization of target DNA was observed after labeling with hematoxylin. Hematoxylin tends to bind to double stranded DNA more avidly than to single stranded DNA. Hence, the presence of complementary DNA was detected easily on the electrode surface with voltammetric measurements.

An electrochemical biosensor was developed to detect target HPV-16 DNA using an anthraquinone (AQ)-labeled pyrrolidinyl peptide nucleic acid (PNA) [60]. This AQ terminated capture probe DNA was immobilized on a chitosan-modified screen-printed carbon electrode. Before hybridization of target DNA with the probe, the redox-active AQ label is close to the electrode surface, which results in high electron transfer to the electrode surface. But after the hybridization, double stranded DNA shows greater rigidity than the probe, so that AQ is separated from the electrode surface and electrochemical signal is reduced. In this way, target HPV-16 DNA was detected down to 4 nM.

An electrical displacement assay was applied to analyze target HPV DNA using an electrochemical detection technology [61]. Dipstick-type microelectrode arrays were fabricated for direct target DNA measurements in sample tubes. Gold electrodes were functionalized with probe DNA, which was later hybridized with ferrocene-modified detection DNA, which was an exact complementary sequence of target DNA and longer than the probe DNA. Hence, when the sensor was dipped inside the sample, target DNA hybridized on the detection DNA and released it from the electrode surface. Employing this approach, target DNA could be measured as a decrease in electrochemical signal. HPV-6 target DNA was measured down to 30 pM using this assay.

An electrochemical sensor was developed to detect the presence of the E6 oncogene from HPV-16 [58]. This biosensor utilized an oligonucleotide capture probe on a

pencil graphite electrode and the degree of hybridization to the E6 gene was recorded as changes in the electrochemical properties of the electrode using voltammetry. This method achieved a LOD of 15 μM.

Immunosensors have also been used to detect the presence of antibodies, avoiding the amplification steps required to detect low viral loads via DNA, as attempted in previously described studies [62]. An interdigitated electrode array covered with an electrolyzed polyaniline-multiwalled carbon nanotube film (PANi-MWCNT) was developed for HPV-16 diagnosis [63]. This electrochemical detection was based on immuno-reaction between an immobilized antigen aptamer HPV-16-L1 on the array surface and an antibody of HPV-16 present in the sample. Antigen/antibody formation reduced the electrical signal of the aptamer due to steric hindrance, which reduced ion-transportation at the polymer-solution interphase. With this biosensor, HPV-16 was measured down to 0.49 nM. Another approach used a conjugated copolymer, poly(HNQ-co-HNQA) to detect conjugation of grafted HPV-16-L1 to anti-HPV antibody in a sample [62]. Steric hindrance due to HPV-16-L1:anti-HPV conjugation changes the rate of cation exchange between the copolymer and the solution as well as the redox kinetics of the copolymer. These electrochemical changes in response to the presence of anti-HPV were then detected using voltammetry.

C. Magnetic Detection Technologies

Magnetic nanoparticle labels have been used to detect HPV genotypes. For instance, target HPV DNA was analyzed at concentrations as low as 10 pM using a giant magnetoresistive (GMR) biochip (see Fig. 9) [64]. Capture probe DNA was immobilized on the chip surface and then biotinylated target DNA was incubated on top of the capture probe. After the hybridization process, streptavidin coated magnetic particle labels were introduced on the chip surface. The electrical resistance of these sensors changes upon binding to target DNA. The GMR sensors are highly sensitive, able to detect changes due to even a single magnetic particle [65]. Using this chip, different HPV genotypes were analyzed. Target DNA was measured by sensing the presence of the magnetic label via the GMR sensor. A similar approach was used in a giant magnetoimpedance (GMI) based biosensor [66], where impedance changes on the magnetic sensor, rather than resistance changes as with the GMR sensor, were used to detect the presence of magnetic labels. With this sensor, HPV-16 and HPV-18 target DNA were analyzed using clinical samples.

More recently, superparamagnetic beads conjugated to biotinylated DNA capture probes specific to HPV-16 were combined with PCR to isolate and detect HPV-16 DNA [67]. Two purification strategies were evaluated: in the indirect purification method, DNA capture probes were

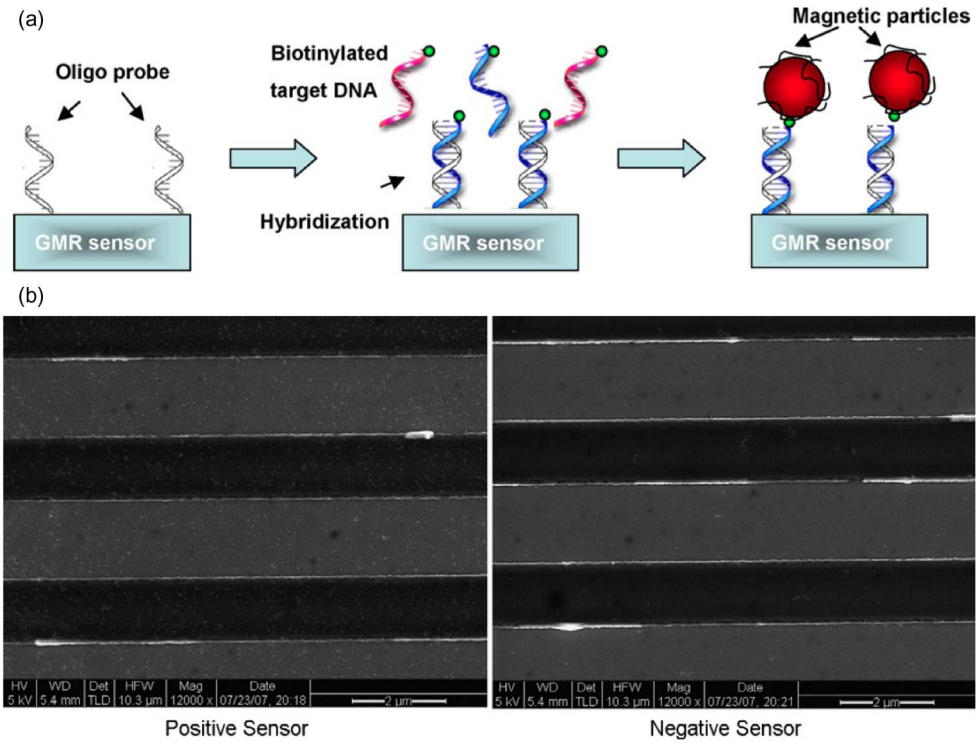

Fig. 9. ***Magnetic-based detection of target HPV DNA on a giant magnetoresistive (GMR) biochip. (a) Steps of the DNA detection protocol. (b) Scanning Electron Microscopy (SEM) micrographs of tests with complementary target DNA and non-complementary target DNA. Reproduced with permission [64].***

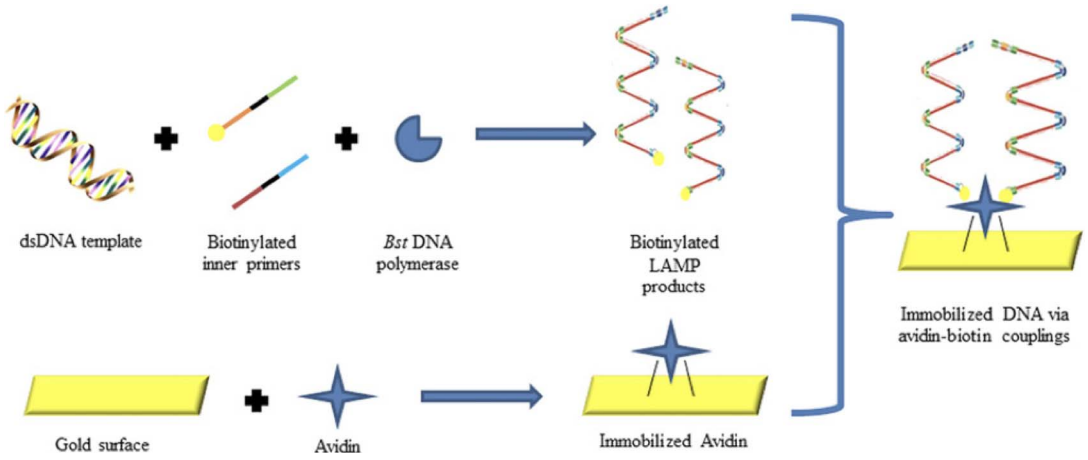

Fig. 10. ***Schematic illustration of target DNA detection on QCM using DNA amplification and biotinylation with LAMP. Reproduced with permission [71].***

conjugated to target DNA and then bound to the magnetic beads; in the direct purification method, capture probes were first bound to the magnetic beads and then hybridized to target DNA. This study found greater success with the direct method, achieving much higher purification efficiency. The efficiency was optimized by tuning the probe density.

D. Mass-Based Detection Technologies

Mass change on a detection surface upon hybridization with target DNA has been utilized for HPV analysis. One study used a piezoelectric sensor with a degenerate probe (with DNA sequences from the conserved region) to detect 16 strains of HPV and two specific probes (with DNA sequences specific to particular strains) to genotype viral strains in cervical scraping samples with a detection limit of 15 nM [68]. Another piezoelectric sensor was developed to detect hybridized target DNA on a sensor surface coated with probe-DNA [69]. The sensor was composed of a quartz piece sandwiched between two gold electrodes. Thiol-modified capture probe DNA was incubated on the electrode. The electrodes were used to induce mechanical oscillation on the quartz piece and the change in oscillation frequency due to hybridization with target DNA from the sample was monitored to measure hybridized target DNA. In this report, HPV PCR amplicons were analyzed using this biosensor.

Another study developed a piezoelectric quartz crystal microbalance (QCM) to identify 11 high-risk genotypes of HPV (types 16, 18, 45, 33, 39, 51, 52, 56, 58, 59, and 68) from PCR products [70]. The same group also developed a method to detect HPV-58 target DNA down to 100 copies using the QCM technique combined with LAMP, as shown in Fig. 10 [71]. LAMP was used to amplify and biotinylate HPV. The biotinylated product of LAMP was then captured with immobilized avidin on the QCM surface and detected due to the changes in the QCM oscillation frequency. This approach improved the LOD of conventional LAMP methodology by up to 10 times.

A linear and a Bayesian classification model have been developed to classify samples as HPV-positive or HPV-negative on the basis of immunosensor measurements from a quartz crystal microbalance [72]. A training data set of both HPV-16-positive and HPV-16-negative oropharyngeal cancer lysate samples was used and classifiers were developed from feature values to create the classification model.

E. Acoustic Detection Technologies

Surface acoustic wave (SAW) biosensors have been developed to detect biomarkers grafted on a sensor area simply by using acoustic waves, which are highly sensitive to changes in the sensor area. A leaky surface acoustic wave (LSAW) biosensor was used for quantitative detection of HPV DNA [73]. Bis-PNA probe was grafted on the sensor to capture double stranded target DNA. Acoustic waves were confined on the microfabricated sensor surface to detect HPV target DNA hybridized on the surface and quantify the presence of the target DNA by measuring phase shifts. This method had a LOD of 1.21 pM (i.e., 140 copies per mL) without need of PCR. With this sensor, HPV-18 was detected in clinical samples with a positive rate of 97.22%. The signal of LSAW bis-PNA biosensor was increased using RecA protein-coated complementary single stranded detection DNA [74]. A LOD of 1 pM for HPV-18 was reached using this label.

III. DIAGNOSTIC GAPS BETWEEN PRESENT TECHNOLOGIES AND UNMET CLINICAL NEED

Since basic laboratory tools such as serological assays and culturing methodologies are not available, HPV infection

is generally diagnosed using molecular methods such as nucleic acid hybridization and nucleic acid amplification assays [75]. Although nucleic acid hybridization assays such as *in situ* hybridization [76] and Southern blotting [77] have successfully been utilized to detect HPV infection, these techniques are limited by low sensitivity, requirement for large sample volume, and high assay time.

Furthermore, distinguishing HPV genotypes is another challenge for HPV diagnosis and the initiation of treatment. There are several U.S. Food and Drug Administration (FDA) approved assays on the market for detection of HPV. The first FDA-approved assay available, Hybrid capture 2 (hc2), can detect 13 HR-HPV genotypes in one sample and is widely used clinically [78]. The Cervista HPV HR and HPV 16/18 tests can detect 14 high-risk HPV genotypes and types 16/18, respectively [79]. The Cobas test detects 14 high-risk HPV types by automatically extracting and amplifying DNA, followed by real-time detection, and is also capable of detecting types 16 and 18 specifically. This test has been clinically validated [80]. The Aptima HPV assay detects the presence of E6 and E7 viral oncogene messenger RNA for high-risk HPV genotypes, which are closely associated with cervical cancer [81]. The Roche Linear array is currently the leading diagnostic tool for genotyping HPV strains, but has limitations of low throughput and objective assessment of results [31]. Many other tests claim to provide improved sensitivity and accuracy, but have yet to be validated and approved by the FDA. One diagnostic tool, the PreTect HPV-Proofer, is available primarily in Europe. One study indicated that the HPV-Proofer has a lower sensitivity than the hc2 test (78.1% versus 95.8%, respectively) and higher specificity (75.5% versus 39.6%, respectively), highlighting the potential advantage of pursuing alternative diagnostic technologies for clinical applications [82]. These assays also largely fail to achieve high throughput testing with small sample sizes and provide limited genotype-specific information.

Nucleic acid amplification assays utilize microarray technologies, sequencing, and PCR-associated amplification tools. These techniques also have some technical barriers including labor-intensive protocols and expensive infrastructure for POC testing, and they are not able to detect multiple HPV genotypes from one sample. Similarly, HPV viral load quantification, which may provide invaluable information on HPV infection and cervical cancer progression, also faces these technical challenges. In addition to molecular diagnostic strategies and nucleic acid-based approaches, immunocytochemistry assays hold promise for detection of HPV by targeting capsid proteins [83]. However, due to the unavailability of reliable biomarkers, these techniques have limitations for differentiating HPV genotypes in various clinical specimens.

An ideal HPV diagnostic test would 1) differentiate multiple HPV genotypes and detect uncommon types with high sensitivity and specificity, 2) quickly provide reliable results for clinical management and initiation of therapy, 3) require a small sample volume, inexpensive reagents, and basic infrastructure that could be operated in resource-constrained settings, and 4) be validated using gold standard methods and clinical samples. Further, diagnosis and differentiation of HPV genotypes at the early stage would allow early treatment, thus decreasing severity of infection and mortality. Recent advances in microfluidics and lab-chip systems including optical [48]–[50], electrical [84], mechanical [85], and microchip platforms [86]–[88] allow intact pathogens including bacteria and viruses to be captured, thus enabling invaluable opportunities for post-genomic and proteomic analysis.

A primary deficiency with the currently used methods is that they are not amenable to rapid and inexpensive testing in large population groups. In many developing regions, instrumentation for real-time PCR amplification is not available, and technology that will enable sensitive, rapid and, inexpensive POC assays for detecting HPV genotypes is critically needed. Up to now, no intact HPV capture and detection assay exists for the POC setting. Therefore, innovative diagnostic methods and platforms are required for HPV diagnosis in clinics, may be achieved by merging nanotechnologies and sensing methodologies.

IV. FUTURE PERSPECTIVE

The prevalence of HPV infection still remains high in both developed and developing countries despite tremendous efforts for HPV treatment and prevention. HPV-related cancers, albeit dominant in cervix, have been reported in external genitalia such as the vulva, vagina and penis, as well as in the oropharynx, which adds to the complexity of HPV screening. Currently, the Pap smear is the main approach for detection of early lesions of cervical cancer. In conjunction with Pap smears, DNA testing of HPV, especially with the capability of detecting high-risk oncogenic subtypes 16 and 18, significantly increases the sensitivity, which facilitates clinical counseling and follow-up examinations, thus improving treatment outcomes. Drug encapsulating topical gels such as Podofilox(Condylox) are also available on market for treatment of HPV, and developing a better understanding of gel deployment can potentially increase effectiveness of topical HPV gels [89]–[92].

HPV-16 and HPV-18 are the most common and likely the most carcinogenic genotypes, since these genotypes are found in 70–76% of cervical cancer patients in all regions of the world [70]. However, the most appropriate HPV test may also be dependent on which HPV type is functionally relevant in tumor formation. Our group used real-time RT-PCR and PCR to detect HPV-16 oncoprotein expression in ThinPrep cervical cytologic specimens [93], [94]. We found that copy numbers for both DNA and RNA E6 and E7 were increased significantly as the severity of the cervical lesions progressed from low to high grade. A recent study documented that HPV-45 was the genotype most likely to express oncogenic E6/E7 mRNA (93% of high-grade lesions),

followed in descending order by HPV-16, HPV-31, and HPV-18 [71]. Thus, tissues with the HPV-16 and HPV-18 genotypes may not necessarily be the ones that are most prone to develop cervical cancer. Instead, those HR-HPV lesions with E6/E7 oncogene expression may be more likely to progress to cancer. Another consideration in assessing cervical cancer risk based on HPV testing is the multiplicity of HPV infections in a given patient. In this same study, the presence of E6/E7 mRNA was more common in lesions with multiple infections compared to lesions with single infections. Thus, future HPV tests may focus on the level of expression of the oncogenic proteins produced by a given HR-HPV.

Yet another approach to clinical diagnosis of HPV is the use of urine samples as an alternative to blood samples from patients. The vast majority of diagnostic tests available are designed to test blood samples, as the concentration of HPV markers of interest exist at a higher concentration in blood than in urine; thus, urine has not yet been studied extensively for this purpose. However, urine samples are far easier to collect and are more favorable for home diagnostic devices. DNA extraction methods offer the potential to concentrate analytes in urine samples to reach the LOD in technologies described in Section II [95]. Thus, methods for processing urine samples and extracting nucleic acids from the specimen on microfluidic devices have been developed [46], [95].

Although molecular diagnosis of HPV DNA and genotyping is clinically useful and widely available in developed countries, the technological complexity associated with molecular testing inherently prevents its rapid testing at the POC (e.g., community clinics) and routine usage in resource-limited settings. WHO has formed criteria for developing POC assays that are affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end users (ASSURED) [96], [97]. Unfortunately, none of the existing HPV assays can meet these criteria. Thus, a fully integrated, portable and inexpensive device that can detect both HR-HPV DNA and oncogenic E6/E7 mRNA would be ideal for the control and prevention of HPV in developing countries. On the other hand, the FDA-approved Roche cobas HPV test can detect simultaneously HR-HPV-DNAs by using PCR-based DNA amplification with a LOD of 150 copies/mL [98]. However, this test requires expensive instrumentation, which limits its applicability at the POC.

At present, emerging biosensing technologies based on microfluidics and nanotechnology hold great potential to develop POC screening assays [48], [99]–[101]. However, the reported sensing technologies still need to go through vigorous clinical validation, and in some cases further assay refinement. It should be pointed out that technology developers should work closely with clinicians in designing and validating HPV POC testing devices and in ultimately delivering ideal POC assays. The remaining challenges also include sample preparation for target DNA amplification or signal amplification, device automation, and data interpretation. One recent study that leverages a micro-a-fluidic approach demonstrated the feasibility of performing rapid CD4 cell counts on-chip with the aid of a mobile detection system [102]. The advantages offered by this technology include short assay time, device automation and simplified analysis of results, as well as the potential for long-distance data transfer. Thus, a single, fully-integrated POC sensing device, in conjunction with mobile-based tele-diagnostics [102], [103], would significantly strengthen current HPV prevention programs in both resource-rich and -poor settings. ■

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Utkan Demirci is a founder of, and has an equity interest in 1) DxNow Inc., a company that is developing microfluidic and imaging technologies for point-of-care diagnostic solutions, and 2) Koek Biotech, a company that is developing microfluidic *in vitro* fertilization technologies for clinical solutions. His interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

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