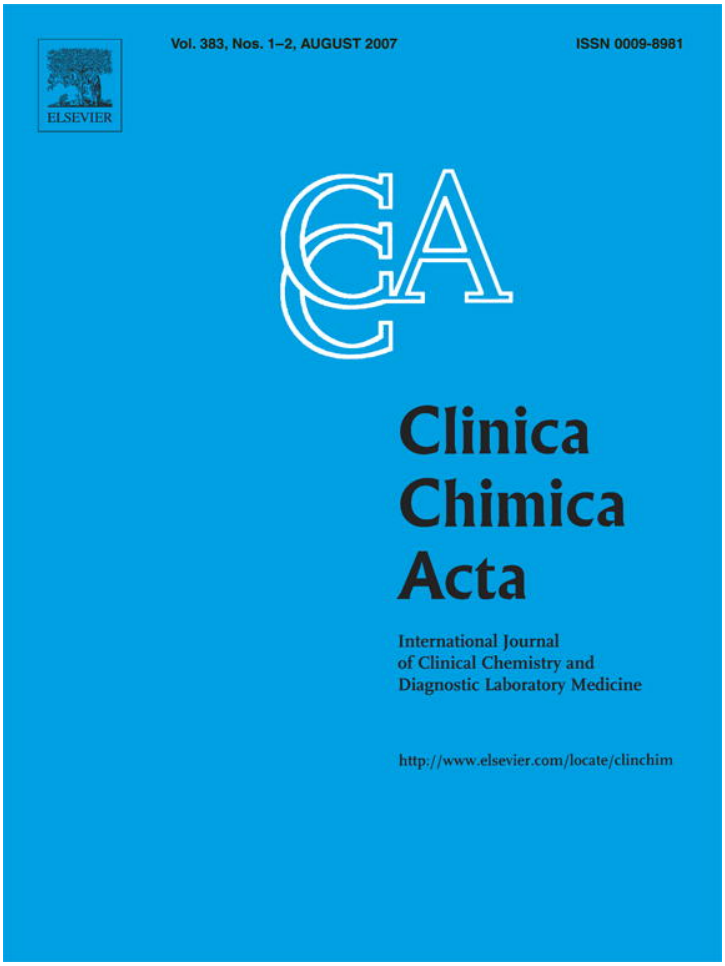

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Development of combined DNA-based piezoelectric biosensors for the simultaneous detection and genotyping of high risk Human Papilloma Virus strains

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Abstract

Background: Human Papilloma Virus (HPV) is a DNA virus belonging to the Papovavirus family. Genital HPV types have been subdivided into medium-low risk, and high-risk (HPV 16 and 18), frequently associated with cervical cancer. Three DNA-based piezoelectric biosensors were here developed for a quick detection and genotyping of HPV.
Methods: We developed a method for the detection and genotyping of HPV in human cervical scraping samples based on coupling DNA piezoelectric sensors with Polymerase Chain Reaction (PCR). The novelty of this work was the design and immobilisation of a degenerate probe (chosen in a conserved region of the viral genome) for the simultaneous detection of 16 virus strains and of two specific probes (chosen in a less-conserved region of the viral genome) for genotyping.
Results: The three biosensors were optimised with synthetic oligonucleotides with good reproducibility (*HPVdeg* $CV\%_{av}$ 9%, *HPV16* $CV\%_{av}$ 9%; *HPV18* $CV\%_{av}$ 11%) with a detection limit of 50 nM. Cervical scraping samples after PCR amplification (in 40–200 nM range), were tested without the need of label with high selectivity and reproducibility. The results were in agreement with a reference method used in routinary analysis.
Conclusion: Piezoelectric biosensors have proven to be suitable for detection and genotyping of HPV.

Keywords: HPV; Quartz crystal; Piezoelectric biosensor; Degenerate probe; PCR; Genotyping

1. Introduction

Biosensors are devices which utilise biological reactions for detecting analytes. Such devices intimately couple a biological recognition element, interacting with the analyte, with a physical transducer that translates the biorecognition event into a useful electric signal. Common transducing elements, including optical [1,2] electrochemical [3–6], or mass-sensitive devices [7,8], generate light, current or frequency signals, respectively.

Because of the increasing interest in the detection of specific DNA sequences using biosensor methods which do not require the use of labels (such as radioisotopes, enzymes, and fluorophores) [9], in the recent years there has been considerable development of label-free DNA piezoelectric biosensors, based on the use of quartz crystal microbalances (QCM). The QCM is an extremely sensitive mass-measuring device (nanograms) which allows dynamic monitoring of hybridisation events, using an oscillating crystal with the DNA probe immobilised on its surface. The increased mass, associated with the hybridisation reaction, results in a decrease of the oscillating frequency.

Applications of this technology include tests for genetic diseases [7,10], detection and characterisations of viruses, bacteria or parasites and assays in the oncogenic field [11].

These systems do not only eliminate the need for labels but also offer the potential advantage of rapid, real-time monitoring of DNA hybridisation, as well as high sensitivity and specificity.

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In this work, we report a biosensor based on DNA detection by piezoelectric sensing for the simultaneous detection and genotyping of a pathogenic virus, the Human Papilloma Virus (HPV). HPV is a double-stranded DNA virus and more than 200 HPV types have been identified [12] among which 40 can infect the genital tract. Genital HPV types have been subdivided into medium-low risk and high-risk (such as 6, 11, 16, 18, 33) which are frequently associated with invasive cervical cancer [13,14]. Furthermore, 93–100% of worldwide invasive carcinomas have been shown to be associated with a limited spectrum of HPV types [15], mostly HPV 16 and 18.

The development and clinical use of HPV vaccines against HPV 16 and 18 makes HPV genotyping a clinical necessity. Various techniques based on molecular detection of HPV DNA are in use, based on the extraction of DNA from the clinical samples followed by PCR amplification and detection of the amplicons for genotyping by hybridisation methods [16–21]. However, detection of viruses by conventional methods, (such as Polymerase Chain Reaction (PCR) based method), is complicated by their high mutation rates. In this regard, primer design is a key feature for the development of successful assays and is performed by using multiple nucleic acid alignments. When detection and differentiation of viral pathogens is required, degenerate primers are often employed in the reaction [22,23]. Thus, primers for virus detection are frequently designed to amplify highly conserved regions, where binding sites are most likely to be retained. By synthesizing primers with degenerate positions, all the possible variants of a target sequence can be covered [24]. Degenerate primers represent a homogeneous mixture of similar, but not identical, oligonucleotides, which have a number of options at different positions in the sequence. In this way a variety of related sequences can anneal to the corresponding complementary sequence.

Similarly to primer design in PCR, probe design in DNA-based sensing is a key step for the success of the analysis [25,26]. In the case of microbial detection, the use of degenerate primers can be transferred to probe design, i.e. use of degenerate probes [27]. With this in mind, our goal was to develop a method for virus identification based on the new probe design for DNA-based piezoelectric sensing allowing simultaneous detection and genotyping of the virus. A degenerate probe is here designed in a conserved genomic region to detect the broadest number of members of the given HPV species. Since in viruses different strains can carry base substitutions even in a conserved region, within the same viral family, the use of degenerate probes allows the simultaneous detection of the presence of different strains, giving yes/no responses for the virus family presence, without any information about the specific viral strain. For this, further characterisation known as genotyping is necessary. To characterise the specific strain (genotype), unique sequences, specific for the particular searched strain, eventually mapping in a close region to the degenerate probe, are employed. By proper combination of degenerate and highly specific sequences, both yes/no and highly specific molecular tests are here designed.

In particular, this work deals with an innovative combined use of degenerate and specific probes to detect and genotype Human Papilloma Virus (HPV) in human specimen. A degenerate probe (designed for detecting several HPV strains, including 6, 11, 16, 18, 33) and specific probes for high risk HPV 16 and HPV 18 were used to develop three different piezoelectric biosensors. Identification and genotyping of real samples (cervical scraping) was achieved and the results proved a clear suitability of the developed multi-array for the detection of the viral strains, since the findings are in agreement with the reference reported method. The proposed approach provides a new appealing tool for important clinical applications. Moreover, this work has a general character since the combined probes design (degenerate and specific) can be coupled to different sensing principles.

Table 1
Sequences of the immobilised probes

Probe	Sequence
HPV 11-mer	5′-biot– ttt gtt act gt –3′
HPVdeg 31-mer	5′-biot- ttt gtt act gt(gt) gt(at) gat ac(ct) ac(at) cgc agt a –3′
HPV16 21-mer	5′-biot- gct gcc ata tct act tca gaa -3′
HPV18 20-mer	5′-biot- ttc tac aca gtc tcc tgt ac -3′

2. Materials and methods

2.1. Materials and reagents

11-mercaptoundecanol and Dextran 500 were from Sigma (Milan, Italy); (+)/−epichlorohydrin and *N*-hydroxysucciminide were purchased from Fluka (Milan, Italy). Ethanol and all the reagents for the buffers were purchased from Merck (Italy).

Two different buffers, immobilisation buffer(NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4) and hybridisation buffer(NaCl 300 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4), were used.

Oligonucleotides were purchased from MWG Biotech (Milan, Italy). The base sequences of the 5'-biotinylated probes are shown in Table 1.

2.2. Piezoelectric biosensor

9.5 MHz AT-Cut quartz crystals (14 mm) with gold evaporated (42.6 mm^2 area) on both sides (International Crystal Manufacturing, USA) were housed in a methacrylate cell, working in static condition. In this set up only one side of the crystal is in contact with the solution in the cell, while the second side is preserved for further uses. The resonance frequency variations caused by changes in mass on the crystal (according to Sauerbrey's equation, [28]) are continuously recorded by the quartz crystal analyzer QCMagic by Elbatech (Marciana, Livorno, Italy), and read directly by a computer connected to the QCMagic interface.

Before use, the crystal was washed in a boiling solution of H_2O_2 (30%), NH_3 (30%) and milliQ water in a 1:1:5 ratio for 10 min and then rinsed with milliQ water. The cleaned gold sensor surface was suitably modified with 11-mercaptoundecanol, Dextran 500 and bromoacetic acid, in accordance with a well established immobilisation chemistry, widely optimised in previous works [10,29]. The so modified crystal is stocked for several days at 4 °C and only before using it is housed into the cell in order to immobilise streptavidin and then a biotinylated probe [10,29].

Once the probe was immobilised on the gold surface, the hybridisation reaction with synthetic oligonucleotides in hybridisation buffer was conducted by adding into the sensor cell 60 μl of the oligonucleotide solution at different concentrations. The reaction was in real-time monitored for 10 or 20 min for the interaction with synthetic oligonucleotides or PCR amplified samples, respectively. The solution was then removed and the surface washed with the same hybridisation buffer to eliminate the unbound oligonucleotides. Fig. 1

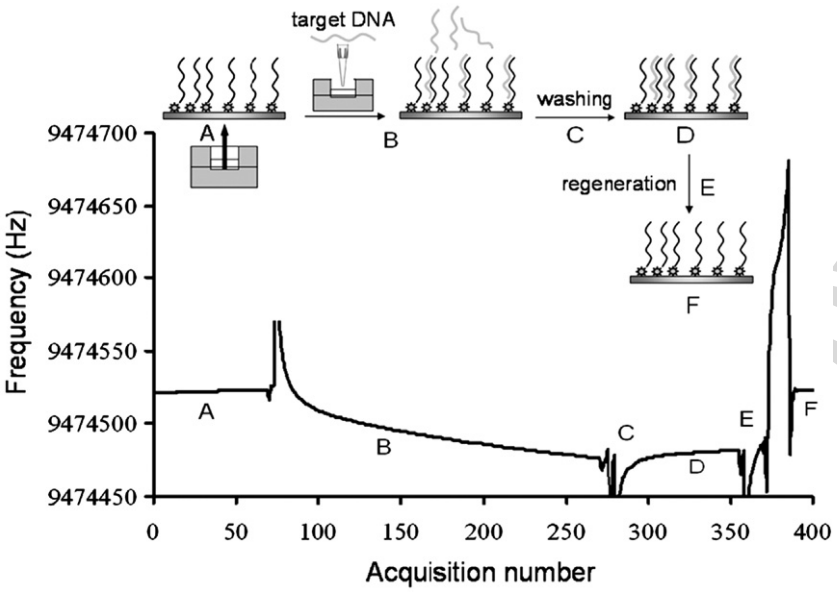

Fig. 1. Schematic diagram of a piezoelectric measurement. Typical sensorgram recorded during a hybridisation reaction. A) The probe is immobilised on the sensor surface. Recording of the baseline in hybridisation buffer. B) Injection of the DNA solution (complementary ssDNA). The dsDNA formation is monitored in real time for 10 or 20 min. C) Removing the DNA solution and washing with hybridisation buffer. D) Baseline in hybridisation buffer. The analytical signal due to the hybrid formation is given by $\triangle F(\mathrm{Hz}) = F_D - F_A$. E) Regeneration (HCl 1 mM) and washing with hybridisation buffer. F) Baseline in hybridisation buffer after regeneration.

shows a typical sensorgram recorded during an hybridisation measurement, by the quartz crystal analyzer which allows to monitor each step of the hybridisation event. We report the frequency shift, related to the hybridisation reaction, as the difference between this final value (F_D) and the value displayed before the hybridisation reaction (F_A). The signal is considered analytically relevant when the $\triangle F > 3$ Hz, which represents three times the blank signal, both for oligonucleotides and PCR samples. After each cycle of hybridisation, the single stranded probe on the crystal surface was regenerated by two consecutive treatments of 30 s with 1 mM HCl (F_E) allowing a further use of the sensor. All the experiments are performed at room temperature.

With this experimental set up all the three biosensors developed in this work were optimised.

2.3. DNA extraction and PCR amplification

Samples, from informed patients undergoing routinely HPV screening at Pisa University Hospital (Italy), were removed from the spatula AYRE and collected in a Falcon tube containing an alcoholic solution. The cells were collected at the bottom of the tube by centrifuging at 3500 rpm for 20 min at room temperature. The pellet was re-suspended in 600 μl of PBS. The DNA extraction was performed using QIAamp DNA mini kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's protocol. Briefly, a digestion with proteinase K was performed for 10 min at 56 °C and, after adding absolute EtOH, the DNA was isolated and centrifuging at 8000 rpm for 1 min. Then two steps of washing and centrifugation using different buffers were performed. Finally, DNA was recovered using 100 μl of H_2O and centrifuged at 13,000 rpm for 1 min.

DNA concentration was estimated spectrophotometrically ($\lambda = 260$ nm) and samples diluted at a final concentration of 10 ng/μl with H_2O.

The primers used to amplify the samples were Hpv18F (5'-gtgtttgctggcataatcaa-3') and Hpv18R (5'-cttattttcagccggtgcag-3') for the amplified products of 353 pb and GP5+ (5'-tttgttactgtggtagatac-3') and GP6+ (5'-gaaaaataaactgtaaatcatattc-3') for the amplified products of 150 pb [10,29].

For the GP5+/GP6+ set of primers we used 50 ng of each DNA sample (5 μl), 5 μl of buffer II 10× (supplied with AmpliTaq Gold; Applied Biosystems, California, USA), 3.5 mM $MgCl_2$, 50 mM of each deoxynucleoside triphosphate, 50 pmol of each forward and reverse primer and 1 U of AmpliTaq Gold in a final volume of 70 μl. Thermal cycler conditions were optimised as follows: initial denaturation for 5 min at 94 °C, followed by 45 cycles of 1.5 min at 94 °C, 2 min at 47.4 °C, and 1.5 min at 72 °C, with a final extension for 7 min at 72 °C.

For the HPV18F/HPV18R set of primers we used the same conditions employed above, except for the thermal cycler conditions: initial denaturation for 5 min at 94 °C; 1 minute at 94 °C, 45 s at 55 °C, 1 min at 72°C for 45 cycles; 7 min at 72 °C.

In order to avoid false negatives due to the absence of DNA and/or to the presence of Taq DNA polymerase inhibitors, a 220 bp region of the β-actin gene was amplified using the following primers: forward 5'-tca tca cca ttg gca atg ag -3' and reverse 5'-cac tgt gtt ggc gta cag gt-3'.

The concentration of the amplified DNA was determined with fluorescence assay by using Picogreen® dye (TD-700 Fluorometer, Turner Biosystem from Analytical Control, Milan, Italy).

2.4. Detection of HPV by PCR and chemiluminescent hybridisation used as reference method

The molecular detection of HPV was performed using the HPV screening L1 (Amplimedical S.p.A Diagnostic Group, Assago, Italy) following the manufacturer instructions. This test is able to give qualitative information about the presence or absence of HPV in the samples. This is achieved by PCR amplification followed by gel electrophoresis. Then the positive samples are processed for genotyping.

To perform the HPV genotyping each sample was denatured at 95 °C for 10 min; then 2 μl of the amplicon were spotted on five different nylon membranes each designated for HPV genotypes 6, 11, 16, 18 and 33. The genotyping of the amplified samples was performed using chemiluminescent probes specific for the HPV 6, 11, 16, 18 and 33.

Each probe was labelled at the 3' end with digoxigenin and subsequently hybridised on the membranes. After hybridisation, membranes were treated with antibody anti-digoxigenin conjugated with alkaline phosphatase and an enzymatic chemiluminescent detection was performed. The signal has been recorded on an autoradiographic film after an over night exposure.

3. Results and discussion

3.1. Selection of the probes to immobilise on three biosensors

The most important step in the design of the assay was to initially develop a biosensor (*HPVdeg*) capable of identifying

the presence or the absence of the HPV virus in the sample without any identification of the genotype.

To obtain this result it was necessary to identify an appropriate probe to be immobilised on the sensor surface.

The sequences of the L1 viral region of five of the most frequent high risk HPV strains (HPV 16, 33, 18, 6, 11) were searched in Genbank. The sequences of the five considered strains were then aligned by using the Clustal W multiple alignment software.

From the alignment it was possible to identify a 11-mer sequence common (conserved) to all the genotypes. Moreover, considering a more extended region it was possible to identify a 31-mer sequence including the conserved 11-mer bases and a 20-mer region with four degeneration points. At these points a base can change into the genotype sequence, differentiating the HPV strains. For example, at the first point of degeneration (base 7241), it is possible to find a thymine or a guanine, depending on the genotype. The 11-mer and the 31-mer sequences were chosen as probe to be immobilised onto the sensor for the development of the *HPVdeg* biosensor.

The 31-mer probe has to be considered a mixture of 16 different sequences (each point of degeneration has two possible options) able to hybridise different HPV genotypes, including the five previously considered genotypes.

If the probe sequence is chosen in the reported region, different sequences in the real sample, corresponding to the broadest number of members of the medium and high risk HPV strains, can anneal to the corresponding complementary probe.

In order to genotype the samples proved to be positive to HPV with the *HPVdeg* sensor, two more biosensors specific for HPV16 (*HPV16*) and HPV18 (*HPV18*) were developed. In this case, the probes to immobilise on the two specific biosensors must be chosen in a region in which the HPV genome is less conserved, to differentiate between the genotypes. In this region only few bases are conserved, but the majority of the sequence is different among the two strains. By choosing as HPV 16 and HPV 18 probes, the sequences reported in Table 1 the specificity of the developed sensors for HPV 16 and HPV 18 is assured.

3.2. Biosensor for the simultaneous detection of different HPV genotypes (HPVdeg)

All the probes were biotinylated and successfully immobilisation, via streptavidin anchoring on the previously modified surface, was achieved, in agreement with previous works conducted with different biotinylated probes [10,29].

Three calibration curves with synthetic oligonucleotides were obtained using the 31-mer probe and the 11-mer probe with their complementary target. The results are shown in Fig. 2. The solution of the 31-mer target, complementary to the 31-mer oligonucleotides probe, has to be considered as a mixture of 16 different sequences. This synthetic sample mimics a real sample containing, eventually, different virus strains.

For each sensor the specificity was tested by adding 1 μM solution of a 26-mer non complementary sequence (5'-ggc aga ggc atc ttc aac gat ggc c-3'), used as negative control. No remarkable hybridisation signal ($\triangle F < 3$ Hz) was obtained, demonstrating the specificity of the sensors. Reproducibility, expressed as average coefficient of variation ($CV\%_{av}$), was evaluated for the different sensors. The measurements were performed in triplicate ($n = 3$).

The highest signals were recorded when the 31-mer probe hybridises its complementary target because of the higher molecular weight (calibration curve A). The lowest signals were observed when the 11-mer probe hybridises the 11-mer target with lower molecular weight (calibration curve C). In between, the hybridisation signals obtained with the 11-mer probe and the 31-mer target were found (curve B). Since the best results were obtained when the 31-mer probe was immobilised (calibration curve A, $CV\%_{av}$ 9%), this biosensor (called *HPVdeg*) with this probe was chosen for the detection of the presence of HPV.

The second step of this work was to investigate the ability of *HPVdeg* to detect the target sequence in PCR samples, from

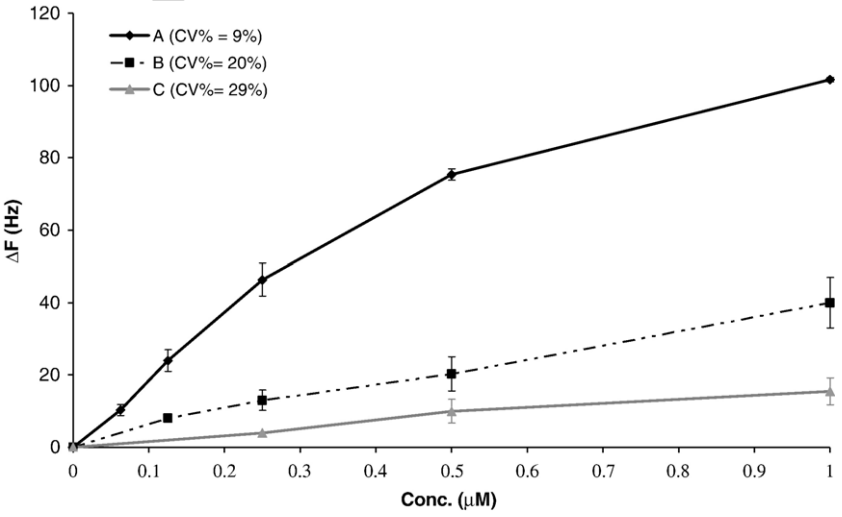

Fig. 2. Calibration curves obtained with A) 31-mer probe/31-mer target; B) 11-mer probe/31-mer target; C) 11-mer probe/11-mer target. Target concentration range: 0.06–1 μM.

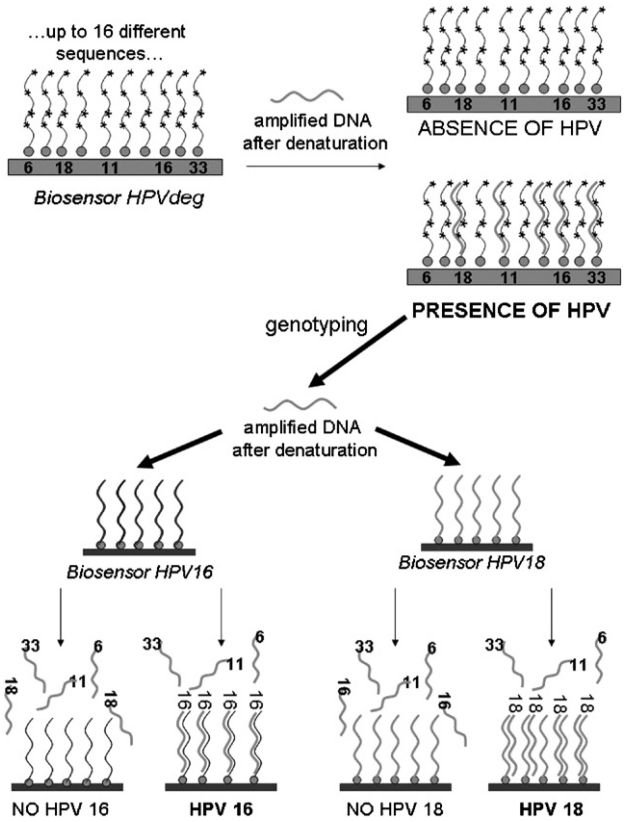

Fig. 3. Schematic description of the developed assay based on combined DNA-piezoelectric biosensors. A) PCR amplified real samples were first processed on *HPVdeg*, in which the degenerate probe is immobilised (* = degeneration point). Samples, positive at first screening, were then processed for genotyping on *HPV16* and *HPV18* biosensors (B).

DNA extracted from cervical scraping. For the analysis with the biosensor, samples, positive at the reference method, were amplified with the forward primer GP5+ and the reverse primer GP6+ in order to amplify all the genotypes potentially present in the sample. The length of these amplified products is around 150 bp, depending on the amplified genotype. All the tested samples resulted in a positive response ($\triangle F\,(n=3)=13\pm3$, 14 ± 2, 23 ± 3 Hz for 3 different samples) indicating the ability of the *HPVdeg* biosensor to detect PCR-amplified DNA. The difference in the recorded signals reflects the concentration variability among the tested samples (concentration range 100–300 nM). To confirm the specificity of the biosensor, blank samples and negative controls (non-specific amplified PCR products) were tested with negligible response ($\triangle F<3$ Hz). The analysis time was 25 min for each sample, considering the hybridisation reaction (20 min) and the washing step to remove the unbound material, before recording the frequency shift. Then the hybrid was dissociated by a fast (30 s) treatment with 1 mM HCl, to allow new measurement cycles.

3.3. Biosensors for the detection of HPV genotype 16 and HPV genotype 18 (HPV16 and HPV18)

After the immobilisation of the specific probes for HPV 16 and HPV 18 (Table 1), the calibration curves for the two sensors were constructed (data not shown) by using the synthetic oligonucleotides. Good reproducibility (*HPV16* $CV\%_{av}$ 9%; *HPV18* $CV\%_{av}$ 11%) and specificity (no remarkable hybridisation signal with a non complementary sequence) were obtained. Since, the final application of the sensors will interest complex matrices containing one or more genotypes at the same time the ability of the sensor to detect the target sequence in the form of PCR amplified DNA was investigated. The biosensor *HPV18* was first tested with PCR samples amplified with primers specific for genotype 18 only (forward primer HPV 18F and reverse primer HPV 18R; amplified product 359 bp). In this way the complexity was stepwise increased from samples containing only HPV 18 to samples containing more than one

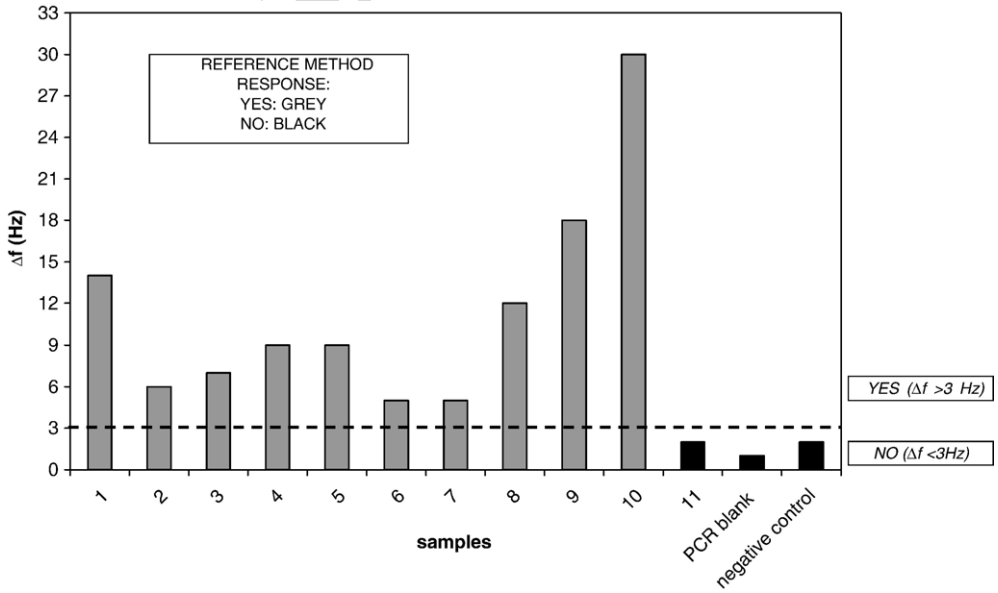

Fig. 4. Detection of the presence or absence of HPV. Signals recorded on the sensor carrying the *HPVdeg* probe with PCR samples amplified with GP5+/GP6+ primers (150 bp), compared with the reference method.

Table 2
Sample identified as positive with *HPVdeg* biosensor, tested with two specific sensors carrying the HPV 16 and HPV 18 probes for genotyping, compared with the reference method

	reference method					Biosensors $\triangle F$ (Hz)	
Sample	6	11	16	18	33	HPV 16	HPV 18
1	X	X	X	X	X	10	4
2	X	X	X	X	X	13	14
3	X	X			X	0	0
4			X			9	0
5			X			16	0
6				X		0	15
7				X		0	11
8			X	X		22	35
9	X	X	X			12	2
10	X	X		X	X	1	12
Negative control						0	1
PCR blank						2	2

HPV strains. These samples (concentration range 50–300 nM) were tested with positive response ($\triangle F(n=3)=18\pm3$, 11 ± 3, 16 ± 2 Hz for three different samples); the results indicate that the biosensor is able to detect the HPV 18 target sequence in amplified products. To confirm the specificity of the biosensor, blanks and negative controls (non-specific amplified PCR products) were tested with negligible response. The experimental detection limit was 30 nM. The sensor was reusable up to 15 times, by dissociating (1 mM HCl for 30 s) the hybrid formed on the surface (data not shown).

3.4. Simultaneous detection and genotyping of high risk Human Papilloma Virus strains

Finally, heterogeneous samples, containing single or different HPV strains or nothing (amplified with the primers "GP5+/GP6+"), were analysed with the three developed biosensors and the results were compared with the reference method. Fig. 3 shows the scheme of the assay.

First, for detecting the presence of HPV (yes/no response) samples were tested with the *HPVdeg* biosensor (Fig. 3A) carrying the 31-mer degenerate probe. The results compared with the reference method are shown in Fig. 4, demonstrating the very good agreement between the two methods. Samples giving signals lower than 3 Hz are considered negative for HPV presence. The differences in the sensor response are due to the heterogeneity of the samples concentration (concentration range: 40–200 nM) and to the different composition with respect to the different strains. The reference method, qualitative as well, also showed variability in the signal intensity (data not shown). The signal is considered analytically relevant when a frequency shift higher than 3 Hz is obtained, which represents three times the blank signal, both for oligonucleotides and PCR samples. Considering this value and experimental detection limit of 40 nM for the PCR samples could be obtained.

For samples proved to be positive from the first screening, with the *HPVdeg* biosensor, further analysis was performed and characterisation (genotyping) of HPV18 and HPV16 strains was simultaneously achieved with the HPV18 and HPV 16 biosensors (Fig. 3B).

Table 2 shows the results, related to samples positive at HPV presence by *HPVdeg*, obtained with the two biosensors, compared with the reference method which simultaneously detects five different genotypes (6, 11, 16, 18, 33). The samples were grouped depending on their strain composition resulting from the reference method. The first group contains samples positive at all the five genotypes (samples 1 and 2); sample 3 contains the strains 6, 11 and 33. The second group is composed of samples containing only genotype 16 (samples 4 and 5) or genotype 18 (samples 6 and 7) or both (sample 8); in the third group samples 9 and 10 contain one of two high risk genotype and some of the others detected by the reference method.

In all the tested samples the biosensors gave coherent responses with the reference method in detecting and genotyping HPV high risk strains 16 and 18. In particular, in the first group HPV 16 and 18 were detected even if other strains were co-present, while in sample 3 none of the two high risk strains was detected. In the samples where only one high risk genotype was present, only the relative biosensor resulted in significant response (samples 4–7 and samples 9–10). Positive responses for both high risk biosensors were obtained for sample 8.

The results demonstrate that by coupling a degenerate probe with highly specific ones it is possible to detect and genotype viruses. This approach could be eventually applied to other molecular-based detection methods, proposed for virus detection and genotyping [19,20,30], especially with high mutation rates [31], where it is difficult to identify a suitable probe with a constant sequence. Actually, the gene-mapping approach uses capture probes of high specificity to each virus and, is of limited utility as a diagnostic tool to be applied to a wide range of viruses.

4. Conclusion

In this paper, the combined use of degenerate and specific probes for DNA-base label free piezoelectric sensing is reported, allowing detection and genotyping of HPV virus strains in human specimen (cervical scraping).

The attractive and innovative aspect of this paper is the combination of the advantages taken from the use of degenerate probes allowing detection of variants of a target sequence (meaning with these, closely related strains in the case of microorganism detection) with the ones of specific probes, used in genotyping. This is the first time, to our knowledge, that this approach is reported for biosensor analysis. With this combined multi-sensor based on three-biosensors (carrying HPVdeg, HPV 18 and HPV 16 probes, respectively) we have demonstrated that simultaneous detection and genotyping of high risk HPV strains can be achieved. In particular, the use of a degenerated probe permits the fast detection of the virus presence, without identifying the particular strain allowing conducting the subsequent analysis for genotyping only on samples proved to be positive from the first screening, thus reducing cost and analysis time. Moreover, the proposed approach has a general validity since it could be used for the detection of different microorganisms, bacteria or viruses,

whose detection with highly specific probes is complicated by their high mutation rates.

In addition, the possibility to export this probe design to detect directly different virus strains without any amplification step by other detection strategies such as fluorescence could eventually contribute to go further in the early detection of a wide spectrum of pathogen strains.

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