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Development of impedimetric DNA sensor for diagnosis of Human
Papillomavirus type 18 infection

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DEVELOPMENT OF IMPEDIMETRIC DNA SENSOR FOR DIAGNOSIS OF HUMAN
PAPILLOMAVIRUS TYPE 18 INFECTION

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Prof. Dr. Valtencir Zucolotto

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*To my grandmother.
In memory of her smiles, her eyes, and her affections.*

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“Whether one is rich or poor, educated or illiterate, religious or nonbelieving, man or woman, black, white, or brown, we are all the same. Physically, emotionally, and mentally, we are all equal. We all share basic needs for food, shelter, safety, and love. We all aspire to happiness and we all shun suffering. Each of us has hopes, worries, fears, and dreams. Each of us wants the best for our family and loved ones. We all experience pain when we suffer loss and joy when we achieve what we seek. On this fundamental level, religion, ethnicity, culture, and language make no difference.”

Tenzin Gyatso, the 14th Dalai Lama

ABSTRACT

CORRER, WR. **Development of impedimetric DNA sensor for diagnosis of Human Papillomavirus type 18 infection.** 2014. 88p.Dissertation (Master in Science) - Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2014.

Currently, the most common strategy employed to detect DNA sequences is PCR (Polymerase Chain Reaction). Nevertheless, in the last few years research on DNA biosensors has increased significantly. Such sensors represent an alternative to PCR in the detection of specific DNA sequences, once they exhibit fast response, low limits of detection, and require simpler sample preparation. The development of a biosensor for detection of DNA from Human Papillomavirus type 18 is reported. To immobilise DNA probe onto indium-tin oxide (ITO) electrodes, a silanisation was carried out using 3-Aminopropyltriethoxysilane (APTES). Silanisation was studied and optimised using ultra-violet absorption spectroscopy, atomic force microscopy, fluorescence microscopy, and cyclic voltammetry. After immobilisation, the hybridisation with target sequence is detected by changes in surface properties of ITO electrode by Cyclic Voltammetry and Electrochemical Impedance Spectroscopy, using the Ferri-Ferrocyanide redox couple. The detection of synthetic target sequence was performed in the range of 12.5 to 100 nM, and 300nM for PCR products. The sensor did not show significant response for non-complementary sequence at 50 nM. This sensor can be applied for fast and low cost detection of HPV genetic material at nanomolar levels.

Keywords: DNA sensor. HPV. Electrochemistry.

RESUMO

CORRER, WR. **Desenvolvimento de um sensor de DNA impedimétrico para o diagnóstico de infecção por Papilomavirus Humano tipo 18.** 2014. 88p. Dissertação (Mestrado em Ciências) - Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2014.

A estratégia mais empregada atualmente na detecção de sequência de DNA é a PCR (Reação em Cadeira da Polimerase). Contudo, nos últimos anos, a pesquisa em biossensores de DNA tem aumentado significativamente. Estes sensores representam uma alternativa a PCR na detecção de sequências específicas de DNA, uma vez que exibem resposta rápida, baixos limites de detecção e requerem preparação simples da amostra. Nesta dissertação descrito o desenvolvimento de um biossensor para a detecção do DNA do Papilomavirus Humano tipo 18. A fim de imobilizar a sequência de captura de DNA em eletrodos de óxido de estanho e índio (ITO), realizou-se uma silanização usando 3-Aminopropiltrietoxissilano (APTES). A reação de silanização foi estudada e otimizada através das técnicas de Espectroscopia de Absorção Ultravioleta, Microscopia de Força Atômica, Microscopia de Fluorescência e Voltametria Cíclica. Após a imobilização, a hibridização com a sequência alvo é detectada através de alterações nas propriedades de superfície do eletrodo através de Voltametria Cíclica e Espectroscopia de Impedância Eletroquímica, usando o par redox Ferri-ferrocianeto. A detecção da sequência alvo sintética foi realizada no intervalo de 12.5 a 100 nM, e para o produto de PCR, 300 nM. O sensor não demonstrou resposta significativa para sequência não complementar a 50 nM. Este sensor pode ser aplicado na detecção rápida e de baixo custo de material genético do HPV a níveis nanomolares.

Palavras-chave: Sensor de DNA. HPV. Eletroquímica.

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1 Introduction

1.1 Cancer and Infectious Diseases in modern Public Health

The beginning of 21st century has been marked by a wide variety of outbreaks: Avian Influenza (H5N1), Swine Influenza (H1N1), Dengue, Cholera, and more recently, Ebola. Nevertheless, innovation and advances in science as well as the dissemination of information by specialized international entities has allowed a rapid and effective outbreak control. The availability of information is, probably, one of the most important tools in modern public health, where World Health Organisation (WHO) develops a central role. For example, before 1990 there was not consistent source of global information about injuries and risk factors for infectious diseases. To overcome this gap, the World Bank and the WHO, in 1991, started a collaborative study named Global Burden of Disease.(1) In 1998, the Centre for Disease Control and Prevention (CDC) developed a comprehensive plan for the prevention and control of emerging infectious threats.(2) Government, researchers, universities, and hospitals provided with these studies and information can plan and develop actions for the effective control of diseases and outbreaks.

Before the use of antibiotics and public programs in vaccination, infectious diseases used to be the leading cause of death. Despite the success in treatment and control of such diseases, they still represent a main public health issue worldwide. Lower respiratory infections, HIV/AIDS and diarrhoeal diseases figure among the top ten causes of death worldwide.(3) The WHO estimates that 57 millions of deaths occurred worldwide in 2013, of which 13 million (22.8%) have been caused by infectious diseases. According to the same report, while the major cause of death is attributed to coronary diseases, which are responsible for roughly 3 in 10 deaths in the globe, countries with low and medium-income still present a great mortality rate resulting from infections. In low-income countries, the top three causes of death are, respectively, lower respiratory infections, HIV/AIDS, and diarrhoeal diseases. Malaria occupies the sixth position and tuberculosis the eighth.(4)

Furthermore, in the last 50 years we have witnessed cancer emerging as major concern for governments and researchers. The GLOBOCAN project estimates the incidence, mortality and prevalence, at the national level, of the most common types of cancer on 184

countries.(5) GLOBOCAN is an extensive study about cancer executed every four years by the International Agency for Research on Cancer (IARC), a specialised agency of the WHO.

In GLOBOCAN 2012 study, it was found that there were 32.6 million people living with cancer worldwide within 5 years of diagnosis, 14 million new cases of cancer, and 8.2 million deaths caused by it,(5) being one among the top ten causes of death.(3)

Cancer had been considered a problem of developed countries, although this scenario has changed over the last few decades. Currently, the developing countries also present a high incidence of cancer, where it is the third or fourth more common cause of death in middle-income countries.(5,3) Fifty seven percent of new cancer cases are diagnosed and 65% of deaths occur in the less developed regions of the world. However, incidence and mortality rates vary globally due to many different risk factors. For example, in developed regions the cancer mortality of men is about 50% of that in less developed regions. In US, cancer is the second most common cause of death and it is expected that, in 2014, about half million of North Americans die due to cancer.(6)

In Brazil, in the last few decades, there has been a change in mortality causes. The occurrence of deaths caused by infectious diseases has diminished while chronic and degenerative diseases have been arising as the major cause of death. It is expected 395,000 new cancer cases just in 2014. Apart from non-melanoma skin cancer, the most frequent cancer types in men are prostate, lung, colon and stomach; in women: breast, colon, cervix and lung.(7)

1.2 Human Papillomavirus (HPV) and Cervical Cancer

Some infectious agents, mainly viruses, seem to strongly influence the development of certain types of human cancer. These viruses are frequently referred to as oncoviruses or oncogenics. Among them, we can highlight HIV, hepatitis B and C, HPV, T-lymphotropic viruses, Epstein-Barr virus (EBV), and herpes virus type 8. According to Parkin,(8) 17.8% of all cancer cases are caused by infectious agents, and this fraction is about 25% in developing regions.

HPV is a double stranded DNA virus which infects squamous epithelia, and are classified according to their risk to develop cancer. Most HPVs are classified as low-risk and produce localized benign warts that do not undergo malignant progression. There are more

than two hundred known types of HPV and the types 16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 are known for their carcinogenicity.(8,9) These high risk HPV types are responsible for nearly 10% of all cancers in women and 5% in men.(10)

The most prevalent viral infection of the reproductive tract is caused by HPV. Humans sexually active may be infected anytime in their lives. Cervical cancer is frequently attributed to a persistent HPV infection. In fact, since the 90s it is widely accepted that a persistent HPV infection is a necessary condition for this cancer type.(11,8) Currently, all cervical cancer cases are attributed to infection of certain types of HPV, where HPV16 and HPV18 are responsible for nearly 70% of them.(12) Moreover, high-risk HPV types are also attributed to some types of vaginal,(13) vulvar,(14) anal,(15) and penile(16) cancers.

Cervical cancer is the fourth most frequent and the third greatest cause of death by cancer in women. Figure 1 shows the cervical cancer incidence worldwide in 2012, when half-million new cases were diagnosed. The highest rate of incidence and mortality of cervical cancer is found in Eastern and Middle Africa. The less developed regions present a high occurrence of cervical cancer, being responsible for nearly 85% of new cases diagnosed in world and for 87% of all deaths. In these areas, 12% of all female cancer cases are due to cervical cancer.(5)

HPV was responsible for 4.8% of cancer caused by infectious agents in 2008(12) and it caused more than 250 thousand deaths in 2012,(5) of which 87% occurred in developing regions. In Brazil, it is expected 15.3 cases per 100 thousand women for cervical cancer, resulting in an estimative of 15 thousand new cases in 2014.(7)

The cancer treatment is expensive for governments and hospitals. The more advanced the disease, the higher the expenses with medicines, hospitalisations, surgeries, etc. Furthermore, the early diagnosis of cancer increases the chances of success in treatment, and, consequently, the survival rate. Regarding to cervical cancer, currently it is classified as a preventable cancer since it is caused by HPV infection. Thus, HPV diagnosis is crucial for the screening of a possible infection to avoid future cancer cases. When carried out efficiently and in case of a positive result, this diagnosis will support an adequate treatment.

Figure 1 - Global incidence of cervical cancer cases in 2012. Estimated age-standardised rates per 100,000.

Source: Ferlay, J. et al.(5)

1.3 Methods for HPV Infection diagnosis

Most of HPV types are harmless and only produce benignant dysplasia, as warts, on skin or genital areas. Nevertheless, high-risk HPVs generate lesions in squamous epithelia which may develop into invasive squamous cell carcinoma.(17) Thus, the persistence of a squamous epithelial lesion is the first clinical evidence of a possible HPV infection or dysplasia. The development of such lesions is driven by the changes in host cell due to viral integration and replication, and detailed oncogenesis mechanism, as well as their molecular basis are beyond the scope of this work, and can be found on the review of Münger *et al.*(17)

Despite cytological abnormalities naturally disappear in more than 85% of patients,(18) the persistence of such lesions, also referred to chronic infection, leads to development of invasive cervical cancer.(18,19) Usually, women are infected early in their lives (20) and approximately 20 years are necessary for cervical cancer to develop in women with normal immune systems.(9) Although HPV infection is a necessary condition for cervical cancer development, it is not sufficient. Risk factors include: ageing, smoking, use of oral contraceptive, number of sexual partners, and immunodeficiency(11,21). The early diagnosis of

HPV infection is highly advantageous, because the evolution of cervical lesions can be closely monitored and habits can be changed to avoid cancer or treat it in early stages.

Currently, cervical cancer diagnosis is made by cytological evaluation and visual inspection of mucosa and tissues. The most widely method used for both screening and diagnosis is the cervical cytology screening, commonly known as Papanicolaou Smear Test^{*}. In this test, cervical cells are collected using a proper swab, followed by staining with Papanicolaou stain. Introduced in 1942 and widely modified since then, this staining method uses 4 or 5 staining agents to reveal different cellular structures, depending on fabricant. However, the basic staining is performed in three steps: firstly, haematoxylin solution is used to stain the nuclei; on the second step, cytoplasm is stained by orange-G agent, demonstrating the maturity of keratinised cells; finally a mixture of two or three agents (polychromatic solution) is used to differentiate squamous cells.(22)

After staining, a qualified cytologist[†] observes the cells under a light microscope. Recently, automated imaging analysis has been also used to pre-screen the samples.(23) Cells are classified according to their morphology and abnormalities, using the Bethesda System.(24) For squamous cells, the possible abnormalities are:

- Atypical squamous cells of undetermined significance (ASC-US)
- Low grade squamous intraepithelial lesion (LSIL)
- High grade squamous intraepithelial lesion (HSIL)
- Squamous cell carcinoma

If a squamous lesion is detected, a colposcopy is performed. In colposcopy exam, a specialised binocular microscope (colposcope) is used to perform visual inspections on cervix and vagina. Through analysis vascularisation, shape, size and colour of the lesions, the health care worker can classify them, and, if necessary, demand a biopsy. A workflow of Pap Screen described diagnosis is presented in Figure 2.

^{*} Also known as Pap smear, Pap test or Pap screen

[†]As well as biomedical scientists and/or histopathologists.

Figure 2 - Pap smear workflow. Squamous Lesion includes LSIL, HSIL and carcinoma in situ. ASCUS, atypical cells of undetermined significance; CIN, cervical intraepithelial neoplasia; Adapted from (18)

Source: adapted from Gravitt, P. E. et al.(18)

Despite the decrease in cervical cancer incidence due to Pap Screening, HPV-related cervical lesions are still a problem for health-care systems. Low specificity of screening tests and considerable variability in cervical dysplasia diagnosis are the major flaws of this screening system.(25) Moreover, in case of detection of atypical cells of undetermined significance (ASC-US) in Pap test, a screening for HPV should be performed. Therefore, there is a demand to efficiently detect HPV infections on clinical samples, and several methods are being reported.

Nowadays, there are two biochemical methods used to detect HPV approved by FDA: Digene Hybrid Capture assay (HC2) and polymerase chain reaction (PCR).(18) Besides these, there is still a considerable number of methods that can be used, for example, PapilloCheck®, COBAS®4800 HPV, The Linear array®, CLART® Human Papillomavirus, INNO-LiPA, Clinical arrays® HPV among others that have shown a good performance for the detection of precancerous lesions of the HPV infection.(25,26) In Table 1, the current available methods for clinical diagnosis of HPV infection are presented. This table is based on the review of Abreu *et al.*(25)

The methods PreTect® HPV Proufer and APTIMA® HPV use viral mRNA to perform the detection of HPV infection. As the squamous disease progress towards neoplasia, the level of viral mRNA is increased. Because these methods rely on the mRNA detection during viral replication, the rate of false positive is significantly decreased.

Table 1 - Methods available to HPV diagnosis based on the review of Abreu et al.(25) (G) stands for “able to genotype”, NA: non-applicable

Method	Company	Technique	Features	Observation	Ref
Hybrid Capture® 2	Digene®	DNA Hybridization to labelled RNA probe	Able to distinguish between HR and LR group, although not (G).	The first approved by FDA.	(28)
Cervista® HPV	Hologic Inc.	DNA Hybridization to labelled RNA probe	Pooled detection of 14 HR types. (G) HPV-16 and -18		(28)
NA	NA	Microarray analysis	(G)	Academic work	(29,26)
PapilloCheck®	Greiner Bio-One GmbH	Microarray analysis	(G) 24 types	Does not amplify HPV-35 and -53	(30,31)
NA	NA	PCR-RFLP (Restriction fragment length polymorphism)	Simple and robust. (G)	Academic work	(32)
NA	NA	Abbot Real-Time PCR	Pooled detection of 12 HR types. (G) HPV-16 and -18	Academic work	(33)
Cobas® 4800 HPV test	Roche Molecular System	Real-Time PCR	Pooled detection of 12 HR types. (G) HPV-16 and -18	Clinically validated. Able to quantify	(34)
CLART® Human Papillomavirus 2	Genomica	Biotinylated Primers + microarray	(G) 35 types	Semi-quantitative	(31)
INNO-LiPA	LiPA HPV GT	Biotinylated Primers + Colourimetric assay	(G) 14 types	Some HR types not detected	(35,36)
Linear Array® HPV Genotyping	Roche Molecular System	PCR coupled with reverse line blot hybridisation	(G) 36 types	Equivocal results for some types	(37)
Clinical Arrays® HPV	Genomica	PCR	(G) 35 types	Identify simple infections or co-infections	(38,39)
MCHA	Boehringer Mannheim	PCR + colourimetric hybridisation	5 HR types		(40)
PreTect® HPV Proofer	NorChip	Real-Time PCR	(G) 5 HR types	Gene E6/E7 mRNA detection	(41)
APTIMA® HPV	Gen-Probe	Real-Time PCR	(G) 14 HR types	Gene E6/E7 mRNA detection	(41)

Source: by the author

DNA amplification

Figure 3 - The evolution of cervical disease is related to an increase in the levels of HPV mRNA, which justifies the use of such molecules to early detect neoplasia caused by HPV.

Source: Aptima HPV Assay(27)

Except for the latter two methods, all methods are based on viral DNA detection. Hybrid Capture® 2 (HC2) and Cervista® HPV work on signal amplification based on chemiluminescence and fluorescence, respectively, from a DNA hybridisation event. All remaining tests presented in **Erro! Fonte de referência não encontrada.** are based on DNA amplification. Genotyping is important when diagnosing HPV infection, since some types are harmless and a false positive, in this case, can cause unnecessary treatment for patient. Thus, most of available methods are able to differentiate between high risk and low risk groups, even individually identifying them among several types. The major drawback in the HC2 method is precisely the absence of genotyping.(28) The tests based on real-time PCR can quantify or semi-quantify the viral load in the samples. However, most of them only provide positive or negative response to HPV infection.

Despite the described techniques described above are implemented in health systems and are recognized as efficient, they have some limitations. The poor sensitivity and specificity are some of the disadvantages of Pap smear, the most common test. Also, the time consuming, expensive instrumentation, specific apparatus and requirement of an expert to analyze the data of the conventional and new biomolecular techniques difficult their wide implementation in health systems.(42)

In the low income countries, 85% of new cases of cervical cancer are diagnosed and approximately 87% of deaths occur due to this cancer type. In these countries, there are low specialized personnel and limited sources and, therefore, a need to accessible, low-cost and portable technology, in order to reach the entire population, using minimal amount of sample. In fact, low cost, rapid response and simple methods are required. To overcome this

gap, sensors and biosensors, which present the required prerequisites, may be an alternative to traditional biomolecular methods.

1.4 Biosensors

Research in biosensor is an interdisciplinary field, including areas as physical-chemistry, analytical chemistry, biochemistry, physics, biology, and medicine. The definition of the term biosensor may suffer little variations depending on the expertise of the author. Nevertheless, all definitions agree that biosensor is an analytical device which converts an event detected by a biological recognition element into a quantifiable and processable signal.(43)

These sensors are constituted by biological recognition elements, transducer elements and a signal processor to analyse the signal. Recognition elements typically used in biosensors may be proteins, such as enzymes or antibodies, nucleic acids, cells and receptors or even a tissue. The transducer element is responsible for translating the biochemical signal from the recognition element into a measurable signal. According to the physical-chemical property detected, resulting from the interaction with the bioreceptor,(44) transduction processes can be classified as: optical, electrochemical, thermometric, piezoelectric, magnetic and micromechanical. A scheme of a biosensor is presented in Figure 4.

Figure 4 - Scheme of a biosensor. After recognition by bioreceptor, the signal is transduced and translated to electrical signal for data acquisition.

Source: by the author

The main concepts of biosensors were elucidated in 1962 by Leyland C. Clark with his innovative work on the detection of glucose in human samples from patients with diabetes. The significant advance observed currently in the area of biosensors has been extensive, and the commonly cited advantages of biosensors are: specificity, rapid response, control and automation, low volume of samples required. Beyond doubt, the major advantage is the specificity due to uniqueness and complex recognition event of molecular coupling, as antibodies with their targets or DNA with their complementary sequence.

1.5 Classification of Biosensors

Typically, biosensors are classified based on the bioreceptor or the transducer, or even on the bioreceptor coupled with the transducer. Several classification schemes can be structured to elucidate all the possibilities.(45)

Bioreceptors are the biological recognition elements or a living biological system, more specifically molecular species that utilizes a biochemical mechanism for recognition. The most common categories of biosensors, based on bioreceptors are: enzymes, nucleic acids/DNA, antibody/antigen, cellular structures/cells and biomimetic materials.

According to the type of transducer, biosensors can also be divided into several categories based on the transduction process, such as electrochemical, optical and mass-based biosensors. These main categories are divided in different subclasses: amperometric, potentiometric, impedimetric and conductimetric for electrochemical transducers; Piezoelectric or magnetoelastic for mass-based transducers; Surface Plasmon Resonance, Surface-Enhanced Raman Scattering, etc for optical transducers. Figure 5 shows a schematic representation of the different classifications of biosensor.

Figure 5 - Classification of biosensors based on their bioreceptor and transducers,. Below, it is highlighted the most common techniques in electrochemical biosensing.

Source: adapted from the reference Velusamy, V. et al.(45)

Source: by the author

1.6 DNA biosensors

DNA biosensors are a subclass of sensors characterised by the use of DNA as a bioreceptor or recognition element. This category of biosensor is currently denominated Genosensor or DNA biosensor. DNA biosensors take advantage of the very specific binding of complementary nucleic-acid sequences, commonly relying on the immobilisation of a single stranded nucleic acid probe on a surface to recognise its complementary nucleic acid target sequence by hybridisation.(46)

The majority of DNA biosensors involve the immobilization of a DNA or DNA-analogue probe, such as Peptidic Nucleic Acid (PNA), onto a transducer surface and the subsequent hybridisation with complementary target sequence. Several types of transduction can be carried out to detect this hybridisation event (Figure 6). Commonly, the amount of target is proportional to the level of hybridization and, therefore, the signal intensity.

Figure 6 - Scheme of a DNA biosensor. After hybridisation, the signal transduction can be carried out by different techniques. Electrical and electrochemical transduction are the simplest because the signal is already electronic, relieving the use of sensors or signal converters.

Source: Drummond, T. G.(47)

DNA sequences have been being manipulated in laboratory for over 50 years and the DNA chemistry is well known. Functional groups can be added at the end of DNA sequences and DNA immobilisation is commonly practiced. There are specialised companies in synthesising DNA sequences with several functionalities. Moreover, DNA is a more stable molecule than protein, its purification from biological or clinical samples is easier, hybridisation is a

fast and reversible event, and the sensor can be regenerated. As a consequence, DNA sensors offer fast detection, are cost effective, simpler, easy to handle and stable.

Despite that, there are several variables that can affect the hybridisation on the substrate surface where probe sequence is immobilised. Physical-chemical properties of substrate, type and length of linkers, density and orientation of probes, type and length of probes and hybridisation conditions are the factors that can influence sensor response.(48)

Currently, there are several methodologies to measure the transduction of the hybridization event. If light is used to detect or produced in the hybridisation, the transduction is made by optical signal, and opto-biochemical techniques can be used. Techniques such as fluorescence,(49) chemiluminescence,(50) and colourimetry(51,52) are common in DNA biosensing.

Surface Plasmon Resonance (SPR)(53) or Surface-Enhanced Raman Scattering spectroscopy (SERS)(54) are some of the emerging methods with high sensitivity and great potential in analytical sciences. The SERS is based on amplification of Raman Effect by a nanostructured metallic surface, where single molecule can be detected,(55,56) mainly to the signal enhancement factor, typically 10^{10} . The resonance of surface electrons at an interface is the basis of SPR technique, where the shift in the angle of an incident light beam is caused by changes in the surface plasmon frequency due to adsorption of molecules. For DNA sensors based on these techniques, subfemtomolar levels of target DNA can be detected.(57)

Mass-sensitive detection, such as quartz crystal microbalance sensors(58) and microcantilever sensors(59) are also applicable to DNA detection. These are based on a shift in vibrational resonance frequency of a piezoelectric crystal or a cantilever, respectively, which can be related to mass adsorption. Detection of femtomolar DNA levels can be achieved with these types of sensor.

Electrochemical biosensors are the most common, perhaps due to their high sensitivity, easy to use, low cost, portability, simplicity, and the possibility of micro fabrication.(60) Currently, their development is one of the largest and fastest growing areas in analytical research. The versatility of electrochemical biosensors comes from the variety of techniques that can be carried out. Therefore, there is a large range of application in analytical detection, including environmental monitoring, food analysis and medical diagnosis.

1.7 Electrochemical DNA biosensors

Electrochemical detection of biomolecules relies on physical-chemical changes generated during recognition by bioreceptors. Changes in electric current, potential or impedance can be measured in recognition process. Moreover, other processes than biorecognition can influence the electrochemical signal, leading to an increase in noise. Examples include, structural/orientation changes, non-covalent interaction and diffusion of molecules.(61)

The DNA probe is a sequence designed to capture the target DNA in solution. Usually, the DNA probe is immobilised onto the electrode surface and subsequently hybridised with complementary target. Detection of DNA is strongly dependent on hybridisation success, and since the DNA is negatively charged, the hybridisation process needs to overcome the electric repulsion between complementary strands. The concentration of counter-ions, the temperature, and the hydrogen bonding are responsible for the stability of DNA pairing.(61) Therefore, the presence of DNA molecules near the electrode surface alters the charge density and accessibility of electrolytes to electrode. As a consequence, electrochemical properties of the electrode are altered and DNA hybridisation can be monitored by electrochemical techniques.

Electrochemical transduction of DNA hybridization can be divided in label-free or labelled methodologies. Usually in label-free methods, the oxidation of DNA bases is detected electrochemically, due to unique electrochemical redox properties of such bases. Therefore, in label-free methods, signal comes from the hybridisation by itself, without any addition of electroactive species.(62) However the electrochemical oxidation of DNA can be affected by hybridisation conditions.(61) DNA-intercalating molecules can be used to contour this problem by generation of their own redox signal.

In labelled detection, redox probes are used and they can be immobilised onto the working electrode, on the referenced probe, or be in solution, as ferro-ferricyanate complex ($[\text{Fe}(\text{CN})_6]^{4-/3-}$). The hybridization between the probe and the target can be electrochemically detected by changes in signal from electroactive label.(62)

1.8 Literature review for HPV electrochemical DNA sensor

Different approaches have been applied for the diagnosis and screening of HPV infection. Disadvantages of traditional methods caused a demand in new technologies, requiring faster and effective results and that can be made available anywhere in the world. Biosensors, more concretely electrochemical DNA biosensors, are devices with a wide application and several advantages. Characteristics like high sensitivity, low cost, portability, small sample volume required, make this type of biosensor an excellent method for diagnosis of HPV infection.

The more recent report in the literature was carried out by Jampasa *et al.*, in 2014.(63) In their report, an electrochemical biosensor for the detection of HPV type 16 DNA was developed using a PNA probe. Screen-printed carbon electrodes were used in Square Wave Voltammetry detection, using anthraquinone (AQ) as redox-active label. Limit of detection was found to be 4nM and their sensor was also tested in real sample for detection of HPV type 16.

Another innovative DNA sensor for HPV16 was described by Campos-Ferreira *et al.*, 2013.(64) In their work, L-cystein was electrodeposited on screen-printed gold electrode. Then, a HPV16 probe was immobilized and measurements were obtained by Differential Pulse Voltammetry, based on the reduction signal of methylene blue (MB), obtaining a detection limit of 18.12 nM.

Genotyping of HPV 16, 18 and 45, was proposed by Civit *et al.*, in 2012,(65) using an electrochemical array. In their work, thiol modified DNA probes were immobilised on an array of 16 gold working electrodes. Detection was carried out in a sandwich format with a horseradish peroxidase-labelled secondary probe by Steps and Sweeps technique. The limit of detection ranged from ca. 100 to 200pM.

In the biosensor developed by Nasirizadeh *et al.*,(66) Cyclic Voltammetry and Differential Pulse Voltammetry were used to detect a 20-mer sequence from L1 gene. An alkanethiol DNA probe self-assembled gold electrode was used to hybridise with target DNA, and electrochemical signal of hematoxylin was exploited, leading to a limit of detection of 3.8 nM.

In 2010, Civit and co-workers(67) reported an electrochemical sensor array for individual and multiplexed detection of two DNA sequences, HPV16E7p and HPV45E6. The de-

tection process was carried out by the reduction of tetramethylbenzidine (TMB) label, using steps and sweeps technique. The limit of detection was obtained in the pM range.

Zari et al, 2009,(68) reported a label-free approach. The DNA hybridised on a screen-printed gold electrode (SPGE) was treated with hydrochloric acid. A limit of detection of 300 fM was achieved measuring the oxidation of released purine bases by Square Wave Voltammetry.

Sabzi and co-workers(69) described the electrochemical immobilisation of a 20-mer probe related to the HPV major capsid protein L1, onto a pencil graphite electrode (PGE). Square Wave Voltammetry measurements were carried out using methylene-blue as a label since it stacks between DNA base pairs, resulting in a limit of detection of 0.5 nM.

In these HPV electrochemical sensors, calibration was performed in the nM range, usually below 200 nM. The reported limit of detection lies from subnanomolar to few nanomolar.

Table 2 - Literature review of electrochemical biosensors for HPV diagnosis

Reference	HPV Bioreceptor	DNA immobilisation	Analyte detection	Transducer	Technique	Calibration Range	LOD	
Jampasa, 2014 (63)	16, L1	14 mer	Lys-NH2	AQ - acpPNA	SPCE	SWV	0.02 to 12 µM	4nM
Campos-Ferreira, 2013 (64)	16	23 mer	-NH2	MB	SPGE	DPV	19 to 250 nM	18.13 nM
	16	24 mer						220 pM
	18	22 mer	-SH, biotinylated	HPR-TMB	4x4 array (16 GWE)	Steps and Sweeps	0 to 50 nM	170 pM
	45	23 mer						110 pM
Nasirizadeh, 2011 (66)	Nucleotide from L1 gene	20 mer	-SH	Haematoxylin	Au Electrode	CV and DPV	12.5 to 350 nM	3.8 nM
Civit, 2010 (67)	16, E7p	154 mer						490 pM
	45, E6	78 mer	-SH	TMB	4x4 array (16 GWE)	Steps and Sweeps	0.5 to 50 nM	110 pM
Zari, 2009 (68)	Specific DNA probe to HPV	20 mer	-SH	Acetate buffer	SPGE	SWV	0 to 5 ng/mL	2 pg/mL
Sabzi, 2008 (69)	Major capsid protein, L1 gene	20 mer	-SH	MB	PGE	SWV	Not informed	0.5 nM

Notes about abbreviations: SPCE (screen-printed carbon electrode); SPGE (screen-printed gold electrode); GWE (gold working electrode); PGE (pencil graphite electrode); MB (methylene blue); TMB (tetramethylbenzidine); HRP (Horseradish peroxidase); DPV (Differential Pulse Voltammetry); SWV (Square Wave Voltammetry). Source: by the author

1.9 Objectives

The strategy for the detection of HPV18 DNA comprises three steps: immobilisation of probe DNA onto working ITO electrode, hybridisation of target HPV DNA, and the electrochemical signal measurement, either voltammetric or impedimetric.

Several methods have been described to achieve DNA immobilisation onto solid substrates.⁽⁷⁰⁾ Among them, silanisation is widely used because the availability of silane molecules with distinct functional groups. In this work an aminosilane (APTES) covalently reacts to ITO electrode to generate active amino groups onto its surface. The cross-linker is used to bind the probe DNA onto the electrode. A detailed description of DNA probe immobilisation is given in section 2.4.1. Charge density and accessibility to electrolyte, among other properties, are affected by the hybridisation of immobilised probe with target DNA. In this work, the electrochemical signal from ferro-ferricyanate complex ($[\text{Fe}(\text{CN})_6]^{4-/3-}$) is used to access the information contained in the surface of ITO electrode. Silanisation and DNA immobilisation are studied and results are shown from section 3.2 and 3.5.

To the best of our knowledge, this work is the first use of ITO electrode and Electrochemical Impedance Spectroscopy for the detection of HPV DNA, as well as the first report of silanisation to achieve immobilisation of HPV DNA.

2 Experimental Section

2.1 Chemicals and Materials

The reagents were used as received, with no further purification (Table 3). All solutions were prepared with ultra pure water, *MiliQ*® process (model MegaUP, Megapurity), with resistivity greater than 18.3 MΩ/cm.

Table 3 - List of reagents used in this work, including their manufacturer and part number.

Material	Part Number	Manufacturer
(3-Aminopropyl)triethoxysilane ≥98.0%	A3648	Sigma Aldrich
Acetone 99.5%	A1017.01.BJ	Synth
Ammonium Hydroxide 28.0-30.0%	221228	Sigma Aldrich
Chloroform 99.8%	C1062.01.BJ	Synth
cis,cis-Muconic acid, 98%	297760025	Acros Organics
Ethanol 99.5%	A1084.01.BL	Synth
Ethidium bromide solution 10 mg/ml in H ₂ O	E1510	Sigma Aldrich
Fluorescein 5(6)-isothiocyanate ≥90.0%	F3651	Sigma Aldrich
Glutaraldehyde 25% in H ₂ O	G6257	Sigma Aldrich
Hydrogen Peroxide ≥29%	P2233.01.BJ	Synth
Potassium Chloride ≥99.0%	P5405	Sigma Aldrich
Potassium hexacyanoferrate(II) trihydrate ≥99.95%	455989	Sigma Aldrich
Potassium phosphate monobasic ≥98.0%	P9791	Sigma Aldrich
Sodium Chloride ≥98.0%	746398	Sigma Aldrich
Sodium Phosphate Dibasic ≥99.0%	S5136	Sigma Aldrich
Toluene 99.9%	8608-2	Mallinckrodt

Source: by the author

Glass slides (25 x 75 x 1.1 mm) coated in one side with Indium Tin Oxide (ITO), with rectangular surface resistivity (R_s) of 8-12 Ω/sq were acquired from Delta Technologies, USA. The slides were cut in 25 x 5 mm sections and used in electrochemical measurements as working electrodes.

Fused silica slides (25 x 75 x 1 mm), GE type 124, were also acquired from Delta Technologies, USA. The slides were cut in 25 x 5 mm sections and used in Ultraviolet Absorbance Spectroscopy.

2.2 Substrate cleaning

The cleanliness and activation of the substrate surface is an essential step in sensor development. The substrates were cleaned as follows:

ITO - After sectioning, ITO electrodes were sonicated using an ultrasound cleaner (UltraCleaner 1600, Unique, Brazil) in the following solvents: acetone, isopropyl alcohol, ethanol and deionised water, 2 minutes each. The last step was repeated, and the ITO slides were dried with nitrogen.

Quartz - After sectioning, quartz slides were cleansed with RCA method. Briefly, a solution containing ammonium hydroxide and hydrogen peroxide in water (1:1:5) was heated to 75-80 °C. The slides were immersed for 10 minutes in this solution. Following that, the slides were immersed for 10 minutes in a solution of hydrochloric acid and hydrogen peroxide in water (1:1:5) at 75-80 °C. The slides were then rinsed in copious amounts of deionised water and dried in nitrogen.

2.3 DNA sequence choice

To determine the target sequence for HPV 18 detection, several available strains genomes were aligned using the open source software *ClustalX* with its standard configuration. Once a conserved sequence is determined, it is useful to test it for hairpin structures and self-dimer formation. It is also necessary to calculate the melting temperature (T_m) of DNA sequence, once this is useful information in DNA hybridisation assays. To perform the calculations, the on-line OligoAnalyser® 3.1 Tool from Integrated DNA Technologies (IDT®, USA) was used. Based on physic-chemical model of DNA molecule, as well as empirical data, this tool is able to calculate several properties of small DNA sequences.

The high specificity of DNA pairing is one of the greatest advantages of genosensors. To avoid nonspecific hybridisations, the target sequence must be aligned against some reference genomes, such as other HPV types and human genome. Moreover, the natural flora of female genital tract is a possible source of DNA contamination during the sample collection. Lactobacilli, Staphylococci, Escherichia, Candida are the most frequent genera in genital flora(71).

The genomes alignments were performed using BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information - NCBI). Briefly, the BLAST is an alignment tool that searches parts of the query in a database and it returns the best scored alignments. It is possible to choose between a protein or nucleotide input, the output can be displayed in different ways, and there are multiple options for optimisation and refinement. The searches were limited to non-redundant nucleotides database (nrBlast), using the possible target as a query. In most cases filters were applied to limit the search to an organism or gender.

2.4 DNA Immobilisation

DNA immobilisation is an import matter in biosensor development, since biosensor sensitivity and selectivity rely on orientation, coverage, surface chemical properties, accessibility, and stability of DNA immobilised probe.(72) In this section it is presented the strategy used for DNA immobilisation as well as the experimental procedures to achieve it.

2.4.1 Strategy

In this section is presented the three main steps towards DNA immobilisation onto quartz slides or ITO electrodes. Firstly, the surface must be cleansed and activated to generate hydroxyl groups (Figure 7, step 1). Thereafter, the surface is chemically activated to generate reactive amino groups, which are used to react with the crosslinker molecules. It is used an organosilane molecule bearing an amino group, as schematised in step 2 on Figure 7. The following step is called cross-linking (Figure 7 step 3), and a bifunctional molecule is used to promote the linkage between the aminosilane and the DNA probe. The final step is the DNA immobilisation onto the activated surface.

Silanisation - After cleaning, quartz slides or ITO electrodes were placed to react with a solution of (3-Aminopropyl)triethoxysilane(APTES). Concentration was varied, as well as silanisation time and solvent, as described in section 2.4.2. This step leads to formation of a silane layer bearing amino groups onto ITO surface.

Cross-linking -Muconic Acid (MuA) or glutaraldehyde were tested as cross-linkers. Cis, cis-Muconic Acid in a concentration of 85.4mM in ethanol reacted with silanised sub-

strates during 1 hour. Glutaraldehyde was allowed to react during the same time, although its concentration was 265 mM in water.

Figure 7 - Scheme of substrate silanisation and crosslinking reactions. **1** ITO or Quartz surface bearing hydroxyl groups reacts with **2** the aminosilane APTES, leading to formation of a silane layer. Such layer contains terminal amino groups, whose are able to react with crosslinker groups. **3** Cis, cisMuconic Acid (MuA) or glutaraldehyde are used as cross-linkers.

Source: by the author

DNA attachment - DNA sequence with 5'-Amino modification (NH_2 -DNA) (Figure 8) was acquired from Integrated DNA Technologies (IDT®, USA) in a synthesis scale of 100 nmol. Further information about sequence choice can be found in section 2.3. Amino modified DNA is also referred to DNA probe, or simply probe, along this dissertation. Activated substrates (Figure 9-1) were allowed to react overnight with 1 μM NH_2 -DNA in PBS 0.15M, pH 7.4. After DNA immobilisation (Figure 9-2), substrates were thoroughly rinsed in deionised water and dried in nitrogen.

Figure 8 - Chemical representation of 5'-Amino modified DNA. The curvy line at the right of the molecule represents any DNA sequence.

Source: Integrated DNA Technologies

storage conditions and sensor shelf lifetime was not tested. Either synthetic target sequence (IDT®, USA) or PCR produced target were tested for detection. Synthetic DNA was diluted in PBS 0.15 M, pH 7.4 in several concentrations. PCR product (2.9 μM) was diluted in PBS buffer 0.15 M, pH 7.4, to 300 nM, heated to 96 °C and rapidly cooled in an ice bath. This heating-cooling process intends to denaturise double stranded DNA, in order to increase hybridisation efficacy onto electrode. Denaturised target DNA is placed to hybridise with immobilised probe for 1 hour at room temperature (Figure 9-3).

Figure 9 - Scheme of the strategy on DNA immobilisation and detection. **1** Surface is activated with amino silane APTES and crosslinker. **2** DNA probe containing amino group at 5' end reacts with crosslinker groups. **3** Target DNA hybridises with probe DNA.

Source: by the author

2.4.2 Optimisation

2.4.2.1 Silanisation reaction time

The influence of silane reaction time on DNA immobilisation was studied. Quartz slides were silanised with 1% APTES (v/v) in toluene for 1, 2, 4, and 6 hours. After thoroughly rinsing with water, quartz slides were placed in a MuA 85 mM solution for 1 hour, and finally

reacted with NH_2 -DNA $1\mu\text{M}$ overnight. UV Absorbance Spectroscopy was used to qualitatively evaluate the immobilised DNA.

2.4.2.2 APTES concentration

Quartz slides were silanised 2 hours under varied APTES concentrations. The APTES concentrations were 0.5%, 1.0%, and 1.5% (v/v), which are equivalent* to 21 mM, 42mM, and 64mM, respectively. After rinsing with water, quartz slides were placed in an 85 mM MuA solution for 1 hour. Finally the functionalised quartz slides reacted with amino modified DNA $1\mu\text{M}$ overnight. Qualitative evaluation of the immobilised DNA was performed using UV Absorbance Spectroscopy.

2.5 Study of the solvent influence on silane layers

Silanisation was performed using four distinct solvents: Acetone, Chloroform, Ethanol, and Toluene. APTES concentration was maintained at 1% (v/v) and silanisation time was fixed to 2 hours. Silane layers were characterised through Fluorescence Microscopy and Atomic Force Microscopy (section 2.8). Moreover, Cyclic Voltammetry measurements were performed to characterise the electrochemical behaviour of the silanised surface.

For Fluorescence Microscopy measurements, silanised quartz slides were placed to react with 1mM Fluorescein Isothiocyanate (FITC) in PBS 150 mM, pH 7.4, during 1 hour. FITC is a fluorophore widely used in molecular biology to mark chemical groups, such as amine and thiol. The molecular structure of FITC is presented in Figure 10 along its excitation and emission spectra. The isothiocyanate group covalently reacts with amino groups, which are present on APTES molecules. Consequently, FITC works as a fluorescent label, marking the regions with amino groups.

The topography and roughness of silanised quartz substrates were evaluated using Atomic Force Microscopy. Topographic data may provide useful information about the mechanism and morphology of silane layer formation.

* Data provided by manufacturer: molecular weight of 221.37 g, density of 1.027 g.ml^{-1} , and purity of 97%

Figure 10 - Fluorescein Isothiocyanate (FITC) molecular structure and Excitation-Emission spectra.

Source: Advanced Imaging Laboratory - University of Victoria – Canada(73)

2.6 Polymerase Chain Reaction (PCR) of HPV 18 target sequence

PCR was performed in a thermal cycler model *Maxygene* (Axygen, USA). The DNA polymerase kit used was *Takara Taq™ Hot Start Version* (Takara Biotechnology, Japan). Primers were acquired from IDT® and HPV 18 genome was acquired from NIBSC (National Institute for Biological Standards and Control, United Kingdom).

HPV 18 target sequence amplification was performed in 50 µl reaction volume, containing Taq buffer, forward primer, reverse primer, genomic template, Taq DNA polymerase, mixture of nucleotides (dNTP) and deionised water. The optimised concentrations and thermal cycle are presented in Table 4. The PCR amplicon was purified using *Wizard® SV Gel and PCR Clean-Up System* kit (Promega, USA). To quantify purified target sequence, a Nano-Drop 1000 Spectrophotometer was used (Thermo Scientific, USA).

Table 4 - Conditions used to amplify target DNA from HPV 18 genome

PCR reagents		Thermal cycle		
Material	Final Concentration	Repetitions	Temperature (°C)	Time (mm:ss)
HPV 18 genome	10 ⁴ units/µl	1	92.0	5:00
Primer Forward	0.4 µM	35 cycles	92.0	0:30
Primer Reverse	0.4 µM		60.0	1:30
Taq DNA Polymerase	1.25 units/50 µl		72.0	2:00
dNTP	0.2 mM each	1	72.0	5:00

Source: by the author

2.7 Electrochemical measurements

Cyclic Voltammetry (CV) was used to monitor surface changes on ITO electrodes. Electrochemical measurements were performed using a potentiostat/galvanostat (MetrohmAutolab, The Netherlands), model PGSTAT 302, with system acquisition and data processing General Purpose Electrochemical System (GPES, version 4.9) and Frequency Response Analyser (FRA, version 4.9).

All electrochemical measurements were performed in Phosphate Saline Buffer (PBS), 0.15M, pH 7.4, at room temperature. To prepare the PBS buffer the following reagent concentrations were used: Potassium Phosphate Monobasic 1.8 mM, Sodium Phosphate Dibasic 10 mM, Sodium Chloride 137 mM, Potassium Chloride 2.7 mM. Additionally, a solution of 1 mM of each, Ferrocyanide and Ferricyanide ($[Fe(CN)_6]^{3/4-}$), was used as electrochemical redox probe. An electrochemical cell (10 ml) with three electrodes set up was used. A square platinum foil (1 x 1 cm) was used as counter electrode. A standard silver chloride (Ag/AgCl/KCl saturated) electrode was used as reference electrode*. ITO electrodes were used as working electrodes.

For silanisation measurements, the potential ranged from -0.1 V to 1.0 V at a scan rate of 0.1 V/s. For sensor construction and calibration, CV was performed between -0.3 V and 0.8 V at a scan rate of 0.1 V/s in 1 mM $[Fe(CN)_6]^{3/4-}$ in PBS 0.15M, pH 7.4, at room temperature (25°C) for sensor construction. No conditioning potential was used nor equilibration time. EIS measurements were performed in a frequency range from 0.1 to 10^4 Hz at an alternated voltage of 0.05 V, centred in open cell potential (ca. 0.2 V). Prior to every EIS measurement, a Cyclic Voltammetry was performed.

*While not in use, this electrode was kept immersed in a saturated potassium chloride solution.

2.8 Characterisation techniques

2.8.1 Ultraviolet Absorption Spectroscopy

Ultraviolet (UV) Absorption Spectroscopy spectra were collected in a U-2900 Spectrophotometer (Hitachi, Japan). Spectra were measured from 200 to 340 nm, using a deuterium lamp as UV source, with a scan speed of 400 nm per minute. For each quartz substrate, a blank spectrum, also referred as background spectrum, was measured prior to any surface modification. A special slide holder was designed to ensure the quality of the spectra and avoid reflection and distortions caused by misplacement of quartz slides inside the spectrophotometer (Figure 11). Substrates were placed in vertical position and perpendicular to the light beam.

Figure 11 - Slide holder designed to use in Absorbance Spectroscopy and Fluorescence Spectroscopy

Source: by the author

2.8.2 Fluorescence Microscopy

Fluorescence Microscopy images from silanised quartz slides labelled with FITC were acquired in a Nikon eclipse TS100 Fluorescence Microscope (Nikon, Japan) equipped with a camera DSRI-1, a 50W mercury light source and filter set EF-4 B-2A, with excitation band pass from 400-440 nm and emission cut-off in 470 nm. The blue line (435 nm) of mercury emission spectrum was used to excite FITC molecule, which shows a peak emission in 521 nm.

2.8.3 Atomic Force Microscopy (AFM)

AFM measurements were acquired in an atomic force microscope, model FlexAFM (Nanosurf, Switzerland), equipped with a 100 μm scan head. Measurements were performed in non-contact mode (also known as tapping mode) using a cantilever with resonance frequency of 190 kHz, model TAP 190AL-G-50 (Tedpella, USA).

The open source software *Gwyddion* version 2.34 was used to process the AFM images, as well as to calculate the roughness parameters. Roughness was evaluated using: average roughness (R_a), mean square roughness (R_q), skewness, and kurtosis. Briefly, R_a measures the average absolute deviation from mean value of a distribution (first momentum); R_q measures the quadratic deviation from the mean value of a distribution (second momentum). Skewness (R_3) is the third momentum of a distribution and it measures the distribution asymmetry. For a normal distribution, skewness is equal to zero. Kurtosis (R_4) is the fourth momentum of a distribution and it describes whether the distribution is broad or sharp, where the kurtosis value for a normal distribution is equal to 3. The equations for each parameter are presented below (Table 5).

Table 5 - Equations for roughness parameters

$R_a = \frac{1}{n} \sum_{i=1}^n x_i - \bar{x} $	$R_q = \left(\frac{1}{n} \sum_{i=1}^n (x_i - \bar{x})^2 \right)^{1/2}$
$R_3 = \frac{1}{nR_q^3} \sum_{i=1}^n (x_i - \bar{x})^3$	$R_4 = \frac{1}{nR_q^4} \sum_{i=1}^n (x_i - \bar{x})^4$

Source: by the author

3 Results and Discussion

3.1 Determination of target sequence from HPV type 18

All HPV types present a double-stranded, circular DNA genome, ca. 8 kilobases in length. The major capsid protein (L1 gene) and the small capsid protein (L2 gene) are used to build the capsid, which protects the virus DNA until it infects a new host cell. E1 to E7 genes codify for viral regulatory proteins, which are responsible for viral genome integration and expression in the host cell. Finally, the last 10% of virus genome is called long control region (LCR) or non-coding region and it holds the origin of replication as well as multiple transcription factor binding sites.(74) To illustrate this genome structure, Figure 12 exhibits the annotation for HPV 18 genes.

HPV 18 Reference Sequence (NC_001357.1 from NCBI) was used as standard for all analyses presented in this dissertation. The first observation when studying HPV is that HPV 18 and 45 (another high risk type) display quite similar genomes. Thus, prior to any target determination in HPV 18, both genomes must be aligned, to determine a non-similar region between those types.

Reference genome is aligned against all other HPV 18, non-reference sequences, to find a conserved region in different strains. Once this probable target sequence is determined, primers are designed to amplify it, as well as the probe sequence. The inset on figure 12 shows the positions of forward primer, reverse primer, target and probe sequences on viral genome. Once primers and probes are determined, its physicochemical properties must be calculated.

OligoAnalyser tool results show a melting temperature (T_m) of 55.6 °C, and no significant self-dimer or hairpin formation for probe sequence (Table 6). Similar results have been found for primers forward and reverse primers (data not shown). Moreover, the selected probe is aligned against certain genera or reign to determine whether DNA from some species could interfere with probe, generating a false positive signal. In Table 6, middle part, the BLAST highest scores for each search are shown. Based only on the score and the occurrence of the organism in vaginal flora, the most likely interfering DNA would be from human

(18/25). On the lowest part of Table 6, it is displayed the sequences names, strings, and their location on genome.

Figure 12 - Scheme of HPV 18 genome: Structural genes, L1 and L2 (→). Viral regulatory proteins, E1 to E7, (→). Inset: Annotation of primers (→), probe sequence (→), and target sequence (→) on original genome strands

Source: by the author

Table 6 - Oligoanalyser and BLAST results for selected DNA probe

OligoAnalyser	name	HPV18p	
	sequence	CTGTATTTGTGTTTGTGGTATGGGT	
	position	7335..7359	
	% CG	40.00%	
	T_m	55.6 °C	
	self-dimer	-1.47 kcal/mol (2 bp)	
	hairpin	all $\Delta G > 0$ at 25 °C	

BLAST	Organism	Specie	Score
	Human		18/25
	Candida	C. dubliniensis	16/25
		C. tropicalis	21/25
		C. albicans	15/25
	Lactobacillus	L. casei	14/25
	Bacteria	Hydrogenobaculum sp.	18/25
	HPV	type 33	10/25
		type 45	11/25

Sequences	Name	Sequence	Location
	Primer F	GCCCTAGTGAGTAACAACCTG	7318..7337
	Probe	CTGTATTTGTGTTTGTGGTATGGGT	7335..7359
	Target	GCCCTAGTGAGTAACAACCTGTATTT GTGTTTGTGGTATGGGTGTTGCTTG TTGGGCTATATATTGTCCTGTATTTC AAGTTATAAACTGCACACCTTACA GCATCCATTTT	7335..7414(c)
	Primer R	AAATGGATGCTGTAAGGTGT	7409..7428(c)

Source: by the author

3.2 Characterisation of the DNA immobilisation

3.2.1 A proof of concept: Initial DNA attachment tests

Prior to any electrochemical experiment, it is necessary to characterise the DNA immobilisation, as well as to optimise it. There is a set of spectroscopic techniques which may be used to investigate DNA immobilisation: UV Absorbance Spectroscopy, ³²P radiolabeling, Ellipsometry, Infrared Reflection-Absorption Spectroscopy (IRRAS), X-ray Photoelectron Spectroscopy (XPS), among others. While most of them demand expensive equipment, UV Absorption Spectroscopy is a practical and time-saving technique. Moreover, DNA exhibits a

260 nm absorbance peak and, consequently, its immobilisation can be monitored using UV Absorption Spectroscopy.

In section 2.4.1, ITO is proposed both as the substrate and the conductive element for sensor construction. However, ITO is deposited on glass, and both exhibit a high absorption below 300 nm. On the other hand, fused silica (or fused quartz) is highly pure (>99.9%) which makes it quite transparent in the UV region, and suitable for optical characterisation of DNA immobilisation. Hydroxyl groups on the substrate surface reacts with silicon from the organosilane molecules, and it is expected that the substrate composition has a low influence on the silane deposition as long as it contains enough hydroxyl groups.(75)

Figure 13 exhibits the UV absorption spectra obtained at each step of DNA immobilisation onto a quartz slide. Although the addition of Muconic Acid (MuA) leads to an absorption increment (—), the reaction with DNA (—) drastically affects the absorption profile, showing the typical DNA absorption peak at 260 nm.

Figure 13 - UV absorption spectroscopy of the steps used in DNA immobilisation.

Source: by the author

In addition, the same slide was immersed for 10 minutes in a 1 mg.ml⁻¹ ethidium bromide (EtBr) solution, followed by washing in deionised water. The slides were illuminated with a UV lamp. Because EtBr intercalates with DNA base pairs and exhibits high fluorescence intensity near 600 nm, it is widely used in molecular biology as a staining agent for direct DNA visualisation.

As shown in Figure 14, the silanised quartz (left) presents fluorescence, where amino modified DNA reacted. It is possible to observe a higher deposition of DNA on the meniscus of the solution, which can be seen as a fluorescent line with high intensity. In the same figure, the control slide (right) had been brought in contact with EtBr as well, however it was

not silanised prior to contact with MuA and DNA. This slide shows no significant fluorescence or absorbance* when compared with the positive one. Thus, there is no significant adsorption of DNA onto quartz slides without silanisation.

Figure 14 - DNA immobilisation onto quartz slide. Left: Fluorescence due to EtBr bound to immobilised DNA. Right: Control slide with no silanisation.

Source: by the author

3.3 Optimisation of DNA immobilisation

After the proof of concept, it was necessary to optimise the conditions of DNA immobilisation. Numerous variables can influence on the yield of this reaction, however the silanisation and cross-linking are essential to establish a proper surface activation.

3.3.1 Silanisation time

According to experimental procedure described on section 2.4.2.1, silanisation time was varied to determine whether it affects the amount of immobilised DNA. Figure 15A exhibits the corrected spectra for substrate absorption (baseline correction). On Figure 15B, it is presented the same spectra with light scattering correction. It is possible to conclude that the higher the silanisation time, the lesser the amount of immobilised DNA. This is counter-intuitive, because one should expect for a higher silanisation time, a higher amount of amino groups available to react with MuA. On the section 3.4.2an explanation for this phenomenon is provided.

*Spectrum not shown

Figure 15 - Time optimisation of DNA immobilisation. (A) UV absorption spectra obtained for 1-6h silanisation time and (B) the same spectra corrected for light scattering.

Source: by the author

3.3.2 APTES concentration

Analysis of APTES concentration was performed to establish whether APTES concentration may affect the amount of immobilised DNA. In Figure 16A, it can be noticed the presence of light scattering as observed on the previous section. Light scattering is probably caused by micro structures formed during silanisation process (Figure 16C), which are not observed on the unmodified region (Figure 16D).

When the spectra are corrected for light scattering (Figure 16B), it becomes evident that APTES concentrations lying in this range seem to play a minor role in the amount of the immobilised DNA. Halliwell et al.(76) reported a factorial analysis on the silanisation conditions, such as surface pre-treatment, reaction temperature, and silane concentration. In their work, mercaptopropyltriethoxysilane (MPTES) was used in concentrations of 2.5 and 5.0 % (v/v), equivalent to 98mM and 196mM, respectively. No significant dependence on silane concentration or reaction temperature with the amount of available thiol groups was reported. Therefore, it is reasonable to suppose that the availability of amino functional groups is not affected by silane concentration, since the silane concentration relies on the mentioned range. Hence, the amount of immobilised DNA shall not be affected as well.

Figure 16 - UV absorbance obtained for optimisation of APTES time. (A) UV absorption spectra measured for 3 APTES concentrations plus negative control; (B) the same spectra corrected for light scattering; (C) Microstructures formed during reaction of APTES in toluene 1% (v/v) during 1 hour. (D) A slide placed in toluene (APTES free) does not exhibit the microstructures.

Source: by the author

The construction of the proposed sensor is based on three reactions: the silanisation using APTES, the binding of MuA as a crosslinker, and the DNA attachment. In this piled up construction, the success of a reaction strongly relies on the success of the previous one, being the silanisation is the most important one. It was observed that increasing APTES concentration and silanisation time led to an increase in light scattering and formation of microstructures, which in turn was responsible for lower levels of DNA attachment.

Besides silane concentration and time of silanisation, the solvent appears to play a subtle yet essential role in SAM uniformity. Although a detailed study of silanisation was not our primary objective, it is extremely necessary, since there is no agreement of none of these variables on the published papers about DNA immobilisation for biosensing (Table 7). APTES concentration varies from 17 mM to 500 mM, and silanisation time ranging from 2 minutes to 72 hours.

Table 7 - Summary of selected references on silanisation using APTES for DNA immobilisation. All silanisation reactions were conducted at room temperature. *mixture of APTES and nBTMS

Reference	Silane	Concentration % (v/v)	mM	Solvent	Time
Lin, 2013(77)	APTES	0.4%	17.1	Toluene	25 min
Cabeça, 2010(78)	APTES	2%	85.4	Acetone	2 h
Zebda, 2006(79)	APTES	-	500.0	Ethanol:water (19:1)	12 h
Moore, 2006(80)	APTES	3%	128.2	Methanol:water (19:1)	30 min
Oh, 2002(81)	APTES*	-	50.0	Toluene	3 h
Marquette, 2002(82)	APTES	10%	427.3	o-xylene	45 min
Joos, 1997(83)	APTES	2%	85.4	Acetone:Water (1:1)	24, 48 or 72 h
Guo, 1994(84)	APTES	1%	42.7	Acetone:Water (19:1)	2 min

Source: by the author

There is a great variety of solvents and experimental conditions used for DNA immobilisation. To better comprehend the role of silane in the posterior steps of sensor construction, it was necessary to further investigate the influence of solvents in silanisation.

3.4 The influence of the solvents in silanisation

In the previous section, it was described how silanisation time and concentration influence the amount of immobilised DNA. A rough silane layer was formed regardless APTES concentration, suggesting that another variable may be responsible for such non-uniformity. Silanised quartz showed a milky appearance and microstructures were observed under optical microscope. Lin et al.(77) and Oh et al.(81) reported the development of DNA sensors, using electrical impedance and fluorescent detection, respectively; however they do not report or refer any work about micro structures formation during silanisation. Up to date, there is no consensus about the solvent, time and concentrations of silanes used for surface modification (Table 7). Nevertheless, some researchers have been applying some efforts towards a better acquaintance on silanisation processes.

In this section, it is described a set of experiments to evaluate the influence of the solvents in silanisation. Fluorescence microscopy is used to qualitatively assess the availability of amino groups from APTES followed by Atomic Force Microscopy results, which demon-

* mixture of APTES and nBTMS

strate the roughness and topology of silanised substrates. Finally, it is shown, by Cyclic Voltammetry, how solvents may influence the electrochemical properties of a silane layer.

3.4.1 Fluorescence Microscopy

A simple and useful experiment is performed to evaluate the silanisation as well as the cross-linking efficacy through fluorescence microscopy. Details of experimental set up are given in section 2.8.2. In a few words, Fluorescein Isothiocyanate (FITC) was used to monitor the presence of amino groups, given its capacity to react with these groups. Thus, keeping the same microscope configuration, it was possible to qualitatively evaluate the silanisation efficacy.

A non-silanised slide was used as a control for fluorescence generated by FITC molecules adsorbed on the quartz surface. It was necessary to add a water droplet on the quartz slide to observe the FITC fluorescence. All solvents successfully promoted silanisation, although ethanol and toluene promoted the highest fluorescence signal, and supposedly, the highest silanisation coverage within 2 hours (Figure 17).

Figure 17 - Fluorescence Microscopy of silanised quartz slides.

Source: by the author

Another set of silanised quartz slides was placed in contact with MuA to react during one hour, as previously described (Section 2.4.1). After a carefully washing, the slides were placed in FITC solution to reveal the presence of amino groups. It is expected that MuA interacts with amino groups, and avoiding reaction with FITC. Thereby no fluorescence should

be observed. Indeed, the entire set of quartz slides used in this experiment (Figure 18) exhibited a low level of fluorescence, except for the slide silanised in the presence of toluene. As observed before, silanisation using toluene as solvent tends to build a thick and rough layer. This may be responsible for entrapping FITC molecules on porous regions and it may lead to a high fluorescence signal.

Figure 18 - Fluorescence Microscopy of silanised quartz slides modified with Muconic Acid (MuA).

Source: by the author

On a third set of silanised quartz slides, glutaraldehyde was used as a crosslinker instead of MuA. Glutaraldehyde is a bifunctional molecule and it is widely used as a fixing agent and crosslinker, because it reacts with amines forming imines. These bonds are strong and stable as long as it does not experience acidic conditions.

After one hour reaction, the fluorescence microscopy results may be seen on Figure 19. The low fluorescence in all slides indicates low availability of amino groups, which demonstrates that glutaraldehyde is more efficient to bind to amino groups than MuA. Indeed, without the proper amino activation using the NHS/EDS chemistry, carboxylic groups react with amino groups forming ionic bonds, which are stable under neutral pH and mild ionic strength. Therefore it is reasonable that slides modified with glutaraldehyde, which forms a covalent bond with amino groups from APTES, exhibits lower fluorescence intensity than MuA modified slides.

Figure 19 - Fluorescence Microscopy of silanised quartz slides modified with Glutaraldehyde.

Source: by the author

3.4.2 Atomic Force Microscopy: roughness caused by silanisation

Atomic Force Microscopy (AFM) has been used to produce topographic images of silanised substrates. While the silanisation time was maintained at 2 hours, the influence of solvent was kept under investigation. Figure 20 exhibits the topographic AFM images in same colour scale for all solvents. Table 8 presents the roughness parameters calculated from AFM images.

Silanisation performed in acetone, chloroform and ethanol has produced similar structures, average roughness (R_a) and mean square roughness (R_q). On the other hand, the silanisation in toluene has shown a more rugged profile (Figure 20D). The calculated mean square roughness for toluene is 2.32 nm, almost four times bigger than the bare quartz. A higher number of structures around 50 nm may be observed (Figure 20D). It may also be observed some larger structures, with sizes lying between 200 and 300 nm, which are not observed for the others solvents.

Table 8 - Roughness parameters calculated from AFM images of silanised substrates and for bare quartz

	R_a (nm)	R_q (nm)	Skewness	Kurtosis
Quartz	0.40	0.69	5.53	76.8
Acetone	0.22	0.49	10.4	170
Chloroform	0.26	0.80	9.67	116
Ethanol	0.44	1.35	11	155
Toluene	1.05	2.32	8.42	101

Source: by the author

Figure 20 - Atomic Force Microscopy (AFM) of silanised quartz slides using distinct solvents. (A) Acetone (B) Chloroform (C) Ethanol (D) Toluene. Colour scale in all images was scaled from 0 to 15 nm.

Source: by the author

The microstructures observed in section 3.3.2 have been also observed in AFM images (Figure 21). The highest structure observed in this figure, nearby the centre of scanned area, is around 7 μm wide and 50 nm high. It is also noticeable the presence of similar structures with variable sizes in the range of micrometers, as well.

Figure 21 - Topographic AFM of silanisation on quartz using toluene as solvent. On this scanning area, it is noticeable the micro structure observed in section §\$ responsible for light scattering

Source: by the author

The microstructures observed in the slides using toluene (Figure 16D, Figure 20D, and Figure 21) are not described in the work of Lin et al.(77), neither in the work of Oh et al.(81), in which toluene had been used as a solvent in silanisation for development of DNA biosensors.

Hu et al.(85) described the structure and morphology of self assembled layers of 3-mercaptopropyltrimethoxysilane (MPTMS), an organosilane which is similar to APTES, using benzene as a silanisation solvent. In their work, MPTMS concentration has been studied, ranging from 5mM to 40 mM, and 30 minutes reaction time. As comparison, the 1% APTES concentration used in this dissertation is equivalent to 42.7 mM, during 2 hours. Hu et al. also described a mechanism (Figure 22) of self-polymerisation of MPTMS molecules, culminating in the formation of submicrometer-sized particles (20-200 nm) onto the substrate surface. According to their work, the degree of self-polymerisation increased with the concentration. As a result, the roughness and disorganisation increases, leading to a small density of thiol groups available on the surface (Figure 22C).

Even though the APTES concentration and silanisation time remained constant, results similar to those found by Hu et al. were observed in our experiments. Toluene and benzene are aromatic solvents and quite similar in structure and polarity. Our results for silanisation using toluene are consistent to those found by Hu et al.

Moreover, their proposed mechanism of layer formation states that few reactive groups are available at high levels of self-polymerisation and the amount of deposited silane is dependent on the reaction time and silane concentration. Therefore, as silanisation time increases, it is reasonable to suppose that self-polymerisation degree increases. Figure 15B (page 58), shows that an increase in silanisation time has led to a lower amount of immobilised DNA. This result may be due to the self-polymerisation, which resulted in fewer amino groups available onto quartz surface.

Figure 22 - Diagram of a mechanism for silane layer growth. MPTMS concentration of (a) 5mM, (b) 20mM, and (c) 40mM. Adapted from Hu et al(85).

Source: by the author

3.4.3 The Influence of solvents on the electrochemical properties of silane layers

In the previous sections, experiments were performed in an attempt to choose the most suitable solvent and conditions for silanisation for DNA immobilisation. While fluorescence microscopy results demonstrate that all solvents promoted surface silanisation, AFM images proved that not all solvents promoted it equally. In fact, the use of toluene has cre-

ated APTES microstructures onto the surface. To conclude this study, cyclic voltammetry was used to determine whether the microstructures influence the electrochemical behaviour of the silanised ITO electrodes.

The APTES modified electrodes were tested in cyclic voltammetry (CV) using 1 mM $[Fe(CN)_6]^{3/4-}$ in PBS 0.15M buffer. The results are presented bellow for the previously described solvents: acetone (Figure 23A), chloroform (Figure 23B), ethanol (Figure 23C) and toluene (Figure 23D). The black line corresponds to the control experiment, in which the ITO electrode was immersed during 1 hour in the respective solvent. The red line was obtained for the same electrode, after silanisation with APTES.

Figure 23 - Cyclic Voltammetry in bare ITO electrodes (red lines) and APTES modified electrodes (black lines) using different solvents: (A) Acetone (B) Chloroform (C) Ethanol (D) Toluene. All vertical axes are in the same scale to facilitate the comparison between solvents.

Source: by the author

The amino group on the silanised electrode is able to participate in charge transfer process, which can increase de catalytic activity, bringing the redox peak potentials closer, and also increasing the cathodic and anodic current peaks.

Table 9 summarises the values of cathodic current peak (I_p^c) and the separation between anodic and cathodic peaks (ΔE_p). It also shows the relative changes of silanised electrode in respect to bare electrode, for both parameters.

ITO silanised using either ethanol or chloroform presented quite similar values for I_p^c and ΔE_p . Additionally, the relative values of ΔE_p are virtually identical. AFM and fluorescence microscopy measurements corroborate this result: there is no significant difference between silanisation using ethanol or chloroform.

When acetone was used as solvent, the silanised electrode exhibited a ΔE_p value of 99 mV, and the I_p^c value was 146 μA , which is 320% higher than the same electrode prior to silanisation. These values evidence a reliable silanisation, in which amino groups are promptly accessible to electrochemical charge transfer.

Table 9 - Cathodic current peak (I_p^c) and the separation between anodic and cathodic peaks (ΔE_p) for APTES modified ITO electrodes. Data obtained from voltammograms displayed on Figure 23.

Solvent	I_p^c (μA)		$\frac{I_p^c}{I_p^{c*}}$	ΔE_p (mV)		$\frac{\Delta E_p}{\Delta E_p^*}$
	Silanised	Control		Silanised	Control	
Acetone	146.4	34.8	4.21	99	279	0.355
Chloroform	62.3	46.2	1.35	144	315	0.457
Ethanol	65.2	38.2	1.70	135	297	0.454
Toluene	71.0	5.8	12.18	117	396	0.295

Source: by the author

The relative ΔE_p for toluene exhibited the lowest value, as well as relative I_p^c exhibited the highest value (Table 9). These changes may indicate a higher modification at electrode surface, which could be interpreted as a higher amount of APTES reacted with ITO surface. On the other hand, it could lead to an erroneous interpretation that toluene promoted the best silanisation. In fact, both voltammograms in the presence of toluene (Figure 23) presented a high capacitive double layer and low redox current peaks. The cyclic voltammetry measurements allied with AFM and FM results, as well as the first DNA immobilisation experiments, shows that the silanisation using toluene has led to the formation of a highly non-uniform and capacitive layer.

On the other hand, silanisation using acetone as solvent has shown low roughness parameters (Table 8 and Figure 20A) and exceptional electrochemical response (Table 9 and Figure 23A). It was decided that silanisation with acetone is the most suitable for the proposed DNA immobilisation. From this point on, all silanisation reactions are performed using APTES 1% (v/v) in acetone.

3.5 Genosensor fabrication

Cyclic voltammetry and Electrochemical Impedance Spectroscopy were used to fully characterise all steps in construction of HPV18 DNA sensor. In the subsection 3.5.1, CV data are discussed focusing on sensor construction. In section 3.5.2, EIS data are presented including a calibration experiment. Finally, blank and non-complementary electrochemical data are presented.

3.5.1 Cyclic voltammetry

Figure 24B displays the cyclic voltammetry for electrode after silanisation (—). As observed before, silanisation caused a decrease in the ΔE_p value and an increase in the redox peak currents. Thereby, the accessible amino groups can thus react with carboxyl groups from the MuA crosslinker.

The voltammogram of crosslinker modification prior to DNA immobilisation is also shown (—). There is a remarkable change in the voltammetric response when MuA is added, where the cathodic and anodic current peaks are decreased. The alternated double bonds should act as a conducting element facilitating the electron transfer between the electrode surface and the redox couple. Conversely, the negative charge of carboxyl groups offers a high potential barrier to be overwhelmed by the electrons. Due to this barrier, electrons are not transferred to the electrode; instead, the reduced form of redox probe accumulates next to the electrode surface building the so called double layer. A schematic representation of this behaviour is presented in Figure 24A and Figure 24C.

The MuA is acting as an insulating layer, hampering the electron transfer between the electrode surface and redox probe. As a result, there is a strong surface blockage* leading to a capacitive response rather than the electrochemical probe current peaks.

* Surface Blockage is a jargon in electrochemistry for the blockage of the electrode surface during charge transfer

Figure 24 - A) Scheme of reduction-oxidation of electrochemical probe. B) Cyclic Voltammetry of ITO surface modification prior to DNA immobilisation: APTES silanised ITO (—) and MuA modified APTES layer (—). C) Scheme of electron hampering caused by MuA immobilisation.

Source: by the author

The addition of amino modified DNA has led to a subtle modification in the voltammetric response of the electrode (Figure 25). The redox probe is quite small when compared to DNA molecule and it does not interact directly with DNA as another electrochemical probes do.(86) It is reasonable to suppose that while the terminal amino group from DNA probe interacts with carboxyl group from MuA, the charge transfer is no longer hampered by the negative charge from the latter. On the other hand, the negative charge of DNA backbone as well as its size can increase the blocking effect.

Figure 25 - Cyclic Voltammetry of NH_2 -DNA immobilisation. Previous steps are maintained to facilitate comparison

Source: by the author

Figure 26 exhibits the cyclic voltammogram of HPV18 target DNA (—) at 50 nM hybridised with probe at ITO electrode surface for 1 hour, as well as the previous steps in sensor construction. The voltammogram obtained for all concentrations of target DNA behave similarly to the one measured for 50nM. The voltammetric response is similar to MuA voltammogram when target hybridises. Since probe DNA is just electrostatically bound to MuA molecules, hybridisation with target may have led to a quasi complete removal of probe from electrode. Then, the hybridisation of complementary DNA molecule destabilised the bound of probe DNA to ITO electrode, and as CV is performed a signal similar to MuA is registered.

Figure 26 - Hybridisation of target DNA after electrode modification. Target DNA significantly alters the voltammetric response of ITO electrode.

Source: by the author

It was tested the sensor response in single strand target concentrations ranging from 12.5 to 100 nM. To compare the different electrodes, the current must be divided by electrode area, in order to express the result as current density. It was observed that the potential of oxidation peak shift to a higher potential, although not in a uniform way, as the target concentration increases. The same is observed for the current density of oxidation peak. Nevertheless, the current density of reduction peak exhibits a linear behaviour with target concentration (Figure 27).

The current density of reduction peak was used to plot the analytical calibration curve, which exhibited a linear response in the range of tested target concentrations (Figure 27). It is noticeable that the greater the target concentration, the higher the intensity of current density of reduction peak. The linear fit was performed in non-zero target concentrations. The blank (section 3.5.3) and full mismatch (section 3.5.4) are exhibited, to contrast to target concentrations values. Linear fit shows a determination coefficient (R^2) of 0.989, and the following equation:

$$J = (0.34 \pm 0.03) \cdot C + (122 \pm 2) \mu A.cm^{-2}$$

where J is the modulus of current density for redox peak, and C is the target DNA concentration. The value for blank sample (0 nM of target) is $123 \mu A.cm^{-2}$, what is in a good agreement with the value predicted by the linear fit model ($122 \mu A.cm^{-2}$).

Figure 27 - Calibration curve using Current Density of reduction peak obtained from Cyclic Voltammetry. Full mismatch at 50 nM and blank (no target) values are also shown.

Source: by the author

3.5.2 Electrochemical Impedance Spectroscopy

Electrochemical Impedance Spectroscopy (EIS) measurements were performed as described in section 0 (page 48). Before each measurement, a cyclic voltammetry was performed to activate the working electrode.

In Figure 28, silanisation using APTES (●) produced a surface with low charge transfer resistance (R_{ct}). The addition of MuA (●) increases the R_{ct} , due to a surface blockage. This corroborates the results found in cyclic voltammetry. Subsequently, probe was immobilised onto ITO electrode (●) and it is observed an increment in R_{ct} value, which means that the probe is also offering blockage to charge transfer between ITO surface and redox couple in solution. Finally, target (●) is allowed to hybridise with probe at ITO surface during 1 hour.

It is noticeable the decrease in R_{ct} value, when compared to probe R_{ct} , therefore the addition of target leads to a change at electrode surface, facilitating the charge transfer. As stated in section 3.5.1, the addition of target may be destabilising the electrostatic interaction between probe and MuA. As a consequence, a fraction of hybridised DNA molecules detach from ITO surface, which reduces the surface blocking effect. The result is a reduction in R_{ct} value tending to the MuA R_{ct} . In Figure 28, target concentration is 50 nM, although regardless target concentration, its signal lies between the MuA and probe EIS spectra.

Figure 28 - EIS measurements for each step in sensor construction and target detection. Regardless target concentration, its signal lies between the MuA and probe EIS spectra.

Source: by the author

One can expect that the higher the target concentration, the higher the detachment of probe DNA from ITO electrode, and, as consequence, higher the change in R_{ct} value. Due

to imperfection in ITO dimensions, R_{ct} values vary to one electrode to another. Although the absolute values of R_{ct} may not be used to evaluate target concentration, the changes in R_{ct} values relatively to probe R_{ct} do. In Figure 29 is presented the calibration curve based on ΔR_{ct} values for each concentration. The resistance changes for blank (section 3.5.3) and full mismatch (section 3.5.4) measurements are also presented.

Figure 29 - Calibration curve of ΔR_{ct} versus target concentration using EIS.

Source: by the author

As in CV analysis, the linear fit was performed in non-zero target concentrations. Linear fit shows a determination coefficient (R^2) of 0.989, and the following equation:

$$\Delta R_{ct} = (2.22 \pm 0.07) \cdot C + (163 \pm 4)\Omega$$

where ΔR_{ct} is the change in charge transfer resistance between probe modification and target hybridisation, and C is the target DNA concentration. The value for blank sample (0 nM of target) is 164 Ω , what is in a good agreement with the value predicted by the linear fit model (163 Ω).

3.5.3 Measurement on blank sample

The electrode for blank measurement was prepared in the same way as the others electrodes. The only difference lies in hybridisation step, where the electrode was placed in PBS buffer free of target DNA. Figure 30 shows the Cyclic Voltammetry and EIS spectra of probe and blank measurement. In CV, the current density of reduction peak was 123 $\mu A \cdot cm^{-2}$ and in EIS, the ΔR_{ct} was 164 Ω . Both measurements are in a good agreement with the respective zero value predicted by linear fitting model.

Figure 30 - Cyclic voltammetry and EIS spectra of blank measurement

Source: by the author

3.5.4 Measurement on full mismatch sample

As in blank sample, full mismatch electrode only differs from other in hybridisation step. The electrode was allowed to hybridise during 1 hour with a non-complementary sequence at 50 nM, followed by CV and EIS measurements. In CV, the current density of reduction peak was $126 \mu A.cm^{-2}$, the same value obtained for 12.5 nM target concentration. In EIS, the ΔR_{ct} was 174Ω , a value closer to blank measurement than to 12.5 nM target. Therefore, EIS may be more suitable to differentiate full mismatch events.

Figure 31 - Cyclic voltammetry and EIS spectra of full mismatch sequence at 50 nM

Source: by the author

3.6 Detection of HPV18 DNA produced by PCR

In previous sections, electrochemical measurements were performed on synthetic single strand target DNA. Nevertheless, HPV is a double stranded DNA virus, and to simulate

genetic material from real sample, a fragment from HPV18 genome was amplified by PCR. In this section, it is shown the PCR data as well as electrochemical measurements.

3.6.1 Polymerase Chain Reaction (PCR) of HPV 18 target sequence

Figure 32 shows the electrophoresis used to confirm the PCR reaction, as described in section 2.6. The first lane contains the DNA ladder (O'RangeRuler 10 bp DNA Ladder, Invitrogen, USA) which is used as a standard for sequence length in gel electrophoresis. After optimization of experimental variables, such as annealing time and temperature, primers concentration, and magnesium chloride concentration (data not shown), the best amplicons are presented on the second and third lanes. These duplicates were obtained under same optimised PCR conditions. The amplicons exhibit a size slightly greater than 100 bp, which is in agreement with the 111 bp of designed target sequence (section 2.3).

From the electrophoresis, it can be noticed that the target sequence was successfully amplified, without formation of secondary or background sequences. This uniqueness is essential for the DNA detection experiments. PCR product was further purified using *Wizard® SV Gel and PCR Clean-Up System* kit (Promega, USA). A Nanodrop Spectrometer was used to estimate the concentration of PCR product. The measured concentration was 206 ng/μL. Considering the 111 bp fragment from virus DNA, the molecular weight of such sequence is close to 68806 g/mol, which lead to an estimated molar concentration of 2.99 μM.

Figure 32 - PCR from HPV18 target sequence. The amplicon band, slightly heavier than 100 bp, is observed with no background or secondary fragments formation.

Source: by the author

3.6.2 Electrochemical measurements of PCR sample

After purification from PCR, target DNA diluted to ca. 300 nM in PBS buffer, 0.15 M, pH 7.4. DNA was heated to 96°C, and then rapidly cooled down in an ice bath. Since target DNA is double stranded, the hybridisation is not favourable to occur at ITO surface. Thus, this heating-cooling procedure intends to denature DNA to favour the hybridisation at electrode. ITO working electrode was placed in a solution containing 300 nM of target DNA in PBS buffer, 0.15M pH 7.4, during 1 hour. After hybridisation, the electrode was rinsed in PBS buffer to remove unspecifically bound DNA molecules.

Figure 33 - Cyclic Voltammetry and EIS spectra of PCR product [300 nM]

Source: by the author

In CV, the current density of reduction peak was $162 \mu A.cm^{-2}$. For 100 nM target concentration the same peak was measured as $158 \mu A.cm^{-2}$. Therefore, PCR target [300 nM] produced a detectable signal, although it is comparable to 100 nM single strand target DNA. In EIS, the difference in charge transfer resistance was 333Ω , which is comparable to 75 nM single strand target DNA (336Ω).

In PCR sample, there is a competition between probes and complementary strand to hybridise with target strand. Despite denaturation treatment, PCR DNA tends to renaturise, which leads to lower signal intensity than the single strand target, due to a small number of available target strands. The results presented in this section demonstrate that the proposed sensor can detect HPV 18 DNA, although a set of experiments should be performed to achieve the suitable specificity and sensitivity for diagnosis. Double strand target at 300nM was enough to induce changes at electrode surface leading to a measurable response. Al-

though, the sensor reproducibility was not high as expected, and several electrodes presented issues at hybridisation step.

4 Conclusion

This work describes the development of a low-cost and fast-response DNA sensor for HPV 18 detection based on immobilisation of amino modified DNA on ITO.

Firstly, HPV 18 target and probe sequences were both designed from alignments of HPV 18 genome and tested for pairing properties and specificity. DNA immobilisation was characterised mainly by UV Absorption Spectroscopy. It was found that the higher the silanisation time the lesser the amount of immobilised DNA. This was attributed to the self-polymerisation of APTES, which tends to increase with time and diminish the amount of available amino groups. Moreover, APTES concentration in the range of 0.5 to 1.5% (v/v) does not significantly affect the amount of immobilised DNA.

However, it was noticed issues in reproducibility of DNA immobilisation. It was observed that the solvent used for silanisation strongly affects the characteristics of silane layer, therefore the subsequent steps. There is no agreement in published papers about silanisation conditions. Hence the role of solvents in silanisation was studied. Fluorescence Microscopy results demonstrate that Acetone, Chloroform, Ethanol, and Toluene can be used as solvents for silanisation, although they promote it at distinct rates and or levels. Atomic Force Microscopy results show that solvents caused the silane layer roughness in this crescent order: Acetone, Chloroform/Ethanol, and Toluene. In silanisation using toluene, it was observed the formation of micro structures. Cyclic Voltammetry was used to electrochemically characterise the silane layer. It was noticed that the higher the roughness of silane layer, the higher the surface blockage effect. Acetone silanisation presented the best electrochemical properties.

Each step in sensor construction was characterised by Cyclic Voltammetry and Electrochemical Impedance Spectroscopy. Amino groups from APTES participate in charge transfer increasing the redox probe current peaks, and therefore lowering the charge transfer resistance (R_{ct}). Muconic Acid promotes the surface blockage, lowering current peaks and increasing R_{ct} , and the immobilisation of amino-modified DNA slightly increases this blockage. A linear response was obtained both in CV and EIS for target concentration in the range of 12.5 to 100 nM.

PCR was optimised to produce the double stranded target sequence. A pure amplicon of approximately 110 bp in length was obtained, whereas the designed target sequence is 111 bp long. The addition of 300 nM denatured target double stranded DNA was enough to produce measurable responses.

The results suggest that this simple method can be applied to fast detection of HPV DNA sequence and with low-cost. Improvement of hybridisation conditions and electro-chemical experimental aspects are underway.

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