INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior de Tecnologia e Gestão

Development and optimization of a biological protocol for DNA detection of *Escherichia coli* O157:H7 by Quartz Crystal Microbalance with Dissipation (QCM-D)

Raquel Oliveira Rodrigues

Final Master project report presented to
Escola Superior de Tecnologia e Gestão
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July 2012

Ao meu marido Sérgio, pelo seu incondicional apoio, amor e motivação. Aos meus pais, família e amigos por serem a minha fonte de inspiração e alegria.

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ABSTRACT

Escherichia coli O157:H7 is a foodborne pathogen associated to outbreaks with high mortality. Since the traditional methods for its detection are often time-consuming, there is a need to develop new techniques that allow a rapid, simple, reliable, specific and sensitive detection. The present study aimed to develop a biological protocol for DNA detection of Escherichia coli O157:H7 using a Quartz Crystal Microbalance with Dissipation (QCM-D), to be applied as a genosensor. DNA thiol Probes and the respective DNA Target were selected from the eae gene of E. coli O157:H7. Several parameters (such as, concentration, incubation time and temperature) were studied and optimized, on the steps of DNA thiol Probes immobilization, 6-Mercapto-1-hexanol (MCH) blocking agent deposition and DNA Target hybridization. Both the DNA probe and target oligonucleotides were linked to fluorochromes allowing the use of Epifluorescence microscopy, to verify the mass deposition results obtained by the QCM-D device, in the gold electrode. In this study, the best results regarding immobilization on the gold electrode at 22ºC were obtained using 1.00 µM of DNA probe and 30 minutes of incubation. MCH blocking agent was prepared with 1x TE buffer instead of Milli-Q water, and best results were achieved with 1.00 mM and 30 minutes of incubation. Co-immobilization of DNA thiol probes with MCH did not reveal a significant improvement compared with the separated steps. An increase in the quantity of target DNA detected in the gold electrode was observed when using 1.00 µM target DNA at 30ºC, however the analysis of the Epifluorescence microscope images indicate that this increase is possibly related with non-specific immobilization instead of hybridization efficiency increase.

KEYWORDS:

Genosensor, Quartz Crystal Microbalance with Dissipation (QCM-D), *Escherichia coli* O157:H7, Epifluorescence microscopy.

RESUMO

Escherichia coli O157:H7 é um microrganismo patogénico associado a surtos alimentares com elevada mortalidade. Considerando que os métodos tradicionais de deteção são demasiado morosos, torna-se necessário desenvolver novas técnicas que permitam uma rápida, simples, fiável, específica e sensível deteção. O presente trabalho teve como objetivo, o desenvolvimento de um protocolo biológico para a deteção de ADN de Escherichia coli O157:H7 usando uma Microbalança de Cristal de Quartzo com factor de Dissipação (QCM-D), de forma a ser aplicado como um genossensor. As sondas tioladas de ADN e, respetivo ADN complementário, foram selecionadas a partir do gene eae de E. coli O157:H7. Foram estudados e otimizados vários parâmetros (concentração, tempo de incubação e temperatura) nos passos de imobilização de sondas tioladas de ADN, de deposição de 6-Mercapto-1-Hexanol (MCH) e hibridação de ADN complementar. Foram adicionados fluorocromos às sondas tioladas de ADN e ADN complementar, para a verificação dos resultados obtidos em massa a partir da QCM-D por microscopia de epifluorescência. Neste estudo, os melhores resultados relativamente à imobilização das sondas de ADN foram conseguidos com 1.00 μM de concentração e tempo de imobilização de 30 minutos. O agente bloqueador (MCH) foi preparado em tampão 1x TE e os melhores resultados foram alcançados com 1.00 mM e 30 minutos de incubação. A co-imobilização de sondas de ADN com MCH não mostrou melhorias significativas. Usando 1.00 μM de ADN complementar a 30ºC, observou-se um aumento da quantidade de ADN depositado no eléctrodo de ouro, contudo as imagens obtidas por microscopia de epifluorescência mostraram que este aumento pode ser devido a imobilização inespecífica e não por aumento da eficiência de hibridação.

PALAVRAS-CHAVE:

Genossensor, Microbalança de Cristal de Quartzo (QCM-D), *Escherichia coli* O157:H7, Microscópio de epifluorescência.

TABLE OF CONTENTS

CHAPTER III - Materials & Methods

CHAPTER IV- Results and Discussion

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

A/E: *Attaching and Effacing mechanism*

DNA: *Deoxyribonucleic acid*

RNA: *Ribonucleic acid*

BLAST: *Basic Local Alignment Search Tool*

***ds*DNA:** *double-stranded DNA*

eae: *E. coli attaching and effacing gene*

EHEC: *Enterohemorrhagic Escherichia coli*

espP: *Extracellular serine protease*

etp: *E. coli tyrosine-protein*

FITC: *Fluorescein fluorochrome*

hlyA: *Hemolysin A*

HUS: *Hemolytic-uremic syndrome*

katP: *Katalase-Peroxidase*

LEE: *Locus of enterocyte effacement*

PCR: *Polymerase chain reaction*

pO157: *Plasmid O157*

QCM: *Quartz Crystal Microbalance*

QCM-D: *Quartz Crystal Microbalance* with *Dissipation*

SAM: *Self-Assembled Monolayer*

***ss*ADN:** *single-stranded DNA*

STEC: *Shiga-like toxin E. coli*

Stx 1 e Stx 2: *Shiga-like toxin 1 and 2*

TxRd: *Texas red fluorochrome*

VTEC: *Verotoxine E. coli*

LIST OF MATHEMATICAL TERMS AND UNITS

a e b: *Crystal depending constants*

A: *Electrode area (m^2)*

f_0 ou f: *Piezoelectrical crystal ressonance (Hz)*

Hz: *Hertz*

M: *Molar (mol/L)*

MHz: *Mega Hertz ($1x10^6$Hz)*

mM: *miliMolar ($1x10^{-3}$ M)*

n: *Faces number in a liquid contact*

ng/cm^2: *Nanograms per square centimeter*

nm: *Nanometers (10^{-9} meters)*

$\bar{x}$: *Average*

Δf: *Frequency variation (Hz)*

Δm: *Surface mass variation (Kg)*

η_L ou η: *Viscosity of liquids (Pa.s)*

η_q: *Viscosity of electrode (Pa.s)*

μm: *Micrometers (10^{-6} meters)*

ρ_L ou ρ: *Density of liquids (Kg/m^3)*

ρ_q: *Density of electrode (Kg/m^3)*

σ: *Standard Deviation*

CHAPTER I- INTRODUCTION

1. MOTIVATION AND STRUCTURE OF THE REPORT

The Center of Disease Control and Prevention (CDC) estimates that 265,000 infections by enterohemorrhagic *Escherichia coli* (EHEC) occur each year, in the United States, with *Escherichia coli* O157 strains causing about 36% of these infections (CDC, 2011 (a)).

E. coli O157:H7 is classified as an EHEC with the ability to cause hemorrhagic colitis, which includes symptoms such as bloody diarrhoea, haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpurea (Doyle *et al*., 1997).

Since the traditional methods to detect foodborne pathogens are often time-consuming (Meng *et al*., 2001) there is a need for new technologies that allows rapid, reliable, simple, specific and sensitive detection (Velusamy *et al*., 2010) for a quick and effective medical intervention, as well as, a rapid eradication of the focus infection diseases.

In the last years, there has been much research activity in the area of biosensors development for detection of microorganism, with different approaches being proposed to assemble the above conditions (Velusamy *et al*., 2010), and that also allows monitoring *in situ* and in real-time, at low cost.

Biosensors based on DNA (genosensors) have recently emerged as one of the most promising biological techniques for the detection of foodborne pathogens, due to, the low cost, reliability and speed detection.

One of the most important steps for the development of genosensors is the optimization of the DNA probe immobilization process and the target hybridization, which enables the specific detection of a foodborne microorganism.

The Quartz Crystal Microbalance with Dissipation (QCM-D) has been recently used, for the detection of the immobilization/hybridization mass phenomena, due to its low cost, robustness and reliability.

The present work report is organized as follows:

In the present chapter, motivation, aims and objectives of the work are presented. In *Chapter II – Bibliographic Review*, theoretical concepts important for this work, namely the ones concerning the bacteria, QCM-D device and epifluorescence microscopy, are described. *Chapter III* concerns the description of materials and methods used to

accomplish this work, with the main results being presented and discussed in *Chapter IV*. Finally, in *Chapter V*, final remarks are made about the work.

2. AIMS AND OBJECTIVES

With the above assumptions, the present project presented to obtain the master degree in Biomedical Technology had as main aims:

- The development and optimization of a biological protocol for DNA detection of *E. coli* O157:H7, on gold electrode surface using QCM-D;
- The optical validation of the developed protocol through the use of fluorescent labels and epifluorescence microscopy.

In order to achieve the proposed aims, the present work had, as objectives:

1. The selection of a specific and small sequence of the *ds*DNA (DNA probe and target) from *E. coli* O157:H7, in order to create a specific genosensor to detect this bacteria strain. To accomplish this objective, some bioinformatic tools were used;
2. The preparation of the functional DNA thiol probe, using a dithiothreitol (DTT) solution, to reduce the disulfide linkage, so the thiol group from the DNA probes could become free and functional;
3. The use of QCM-D device to evaluate the immobilization/hybridization mass phenomena in the gold electrodes, through the study of some parameters, such as concentration and time incubation, in the steps of:
    a. DNA probe immobilization,
    b. Blocking agent deposition,
    c. DNA target hybridization.
4. The evaluation of temperature influence on the efficiency of the DNA target hybridization;
5. The comparison study between the final optimized protocol with the separated steps of DNA probe immobilization, blocking agent deposition and DNA hybridization, and the co-immobilization of the DNA thiol probes with the blocking agent, followed by the DNA target hybridization step.

CHAPTER II- BIBLIOGRAPHIC REVIEW

1. ESCHERICHIA COLI (E. COLI)

Escherichia coli was identified in 1885 by the German paediatrician Theodor Escherich. This bacterium belongs to the *Enterobacteriaceae* family and it is a gram-negative, bacilli, mobile, flagellar, non spore-forming, anaerobic facultative and oxidase negative bacterium (FDA, 2002; Jay, 2005).

This bacterium lives symbiotically in the intestinal tract of warm-blooded animals, including humans, suppressing the proliferation of pathogenic bacteria and synthesizing a considerable amount of vitamins (Olsen *et al.*, 2000). However, there are classes of these bacteria that, due to mutations or genetic material exchanges with other pathogens, become virulent to their hosts.

According to the mechanism of pathogenicity, clinical symptoms, epidemiology and/or cell line phenotype adherence, they are classified in *Enteroaggregative E. coli (EAggEC), Enteroinvasive E. coli (EIEC), Enterotoxigenic E. coli (ETEC), Enteropathogenic E. coli (EPEC) and Enterohemorrhagic E. coli (EHEC)* (FDA, 2002).

E. coli samples can be serotyped and classified by the determination of their antigens, namely the somatic antigen *O*, which characterizes the serogroup, the flagellar antigen *H*, which characterizes the serotype, and capsular antigen *K*. This particular combination of antigens, *O*, *H* and *K*, or just *O* and *H*, as it is commonly used, defines the serotype of the sample. However, serotyping by itself does not give sufficient information from the pathogenic strain, which is usually made by verification tests for the toxin production (Tokarskyy *et al.*, 2008; Bastos, 2009).

1.1. ENTEROHEMORRHAGIC *E. COLI* (EHEC)

Frequently, EHEC foodborne outbreaks are caused by the consumption of sandwiches, undercooked ground beef, unpasteurized milk and juices, water, fruit and raw vegetables (Tokarskyy *et al.*, 2008).

EHEC have the ability to cause hemorrhagic colitis, which includes symptoms, such as bloody diarrhoea, haemolytic uremic syndrome (HUS), in which occurs haemolytic anaemia (red cell destruction), thrombotic thrombocytopenic purpurea, kidney failure (leading to the need for dialysis and kidney transplantation), or even death, especially in children (Doyle *et al*., 1997; FDA/CFSAN, 2001; Bastos, 2009).

Within the class of EHEC more than 60 strains are recognized (Karmali, 1989), with the strain O157:H7 being highlighted, as the most predominant one and often associated with outbreaks of hemorrhagic colitis and HUS (WHO, 2011). In 2002, the Center of Disease Control (CDC) recognized *E. coli* O157:H7 as one of the strains associated to foodborne outbreaks with higher mortality, placing it in the list of microorganisms capable of being used for biological attack purposes (CDC, 2005).

CDC also estimated that *E. coli* O157:H7 causes each year in USA, more than 73,000 cases of human illness, including 2168 hospitalizations and 61 deaths (CDC, 2005), numbers that are still accepted by this government agency in the present days.

1.1.1. E. COLI O157:H7

The main characteristics that distinguish *E. coli* O157:H7 from other strains are the absence or reduced growth at 44°C, the inability to use sorbitol or produce β-glucuronidase enzyme. Therefore, it is impossible to detect this faecal coliform strain by using the Most Probable Number (MPN) method, which uses the lactose fermentation at 44.5 °C, or the direct analysis of *E. coli* using substrates for the production of β-glucuronidase enzyme (Silva *et al.*, 2003). However, the use of these methods in laboratory analysis, allows the differentiation with other strains.

The infective dose is still unknown, however, the Food and Drug Administration (FDA) estimated from a collection of outbreaks investigated in USA, that it may be as low as the one of *Shigella spp*, which is less than 10 organisms per gram of consumed food (FDA, 2009).

The contamination of food, water and environment by this strain has been largely attributed to ruminants, particularly bovine and ovine animals, which often present asymptomatic infections. The intestinal tract of these animals serves as a reservoir and poor sanitation and food processing conditions, can convey in the spread of this enterohemorrhagic bacteria. Nevertheless, there are also recorded cases of dissemination by direct contact person-to-person, by faecal-oral transmission and by direct contact with bovines (Silva *et al*, 2003).

The main clinical manifestations of *E. coli* O157:H7 range from asymptomatic infections, bloody diarrhoea, HUS complications, acute renal failure and death.

Normally, after an incubation period of 1-7 days, severe abdominal pain is followed by diarrhoea. Around 2-3 days later, bloody diarrhoea appears, generally characterized

by the absence of fever, which is not a common symptom at this stage. The more severe complications, such HUS, appear between 2-14 days after the hemorrhagic diarrhoea. Such complications are more common in children and the elderly population (WHO, 2011). A compilation of studies in the USA investigated by Panos and co-workers (2006), predicted that 10-15% of the population infected with *E. coli* O157:H7 can develop HUS and 1.2% will not survive to the infection (FDA/CFSAN, 2001; Panos *et al*., 2006; ECDC, 2011).

1.1.2. E. COLI O157:H7 WORLDWIDE OUTBREAKS

The first detected foodborne outbreak by *E. coli* O157: H7 happened in 1982 and was caused by sandwiches with undercooked ground beef in a USA fast food restaurant. In 1993, a major outbreak involved more than 700 individuals in 4 USA states, with 51 cases of HUS reported and leading to the death of 4 individuals. Also in this outbreak, the focus diseases was found in hamburger's undercooked meat, in a regional network of fast-food restaurants, calling attention for meat as a potential source of this pathogen microorganism (Karmali, 1989; Silva *et al.*, 2003). In the same year, another outbreak was investigated involving 26 USA states, where 102 individuals were hospitalized, 31 individuals had HUS and 3 died. In this case, the incriminated foods were fresh spinach.

In 2009-2011, the recorded number of USA foodborne outbreaks caused by *E. coli* O157:H7 decreased, perhaps due to more restrictive hygienic rules adopted by food and transportation companies. However, in these 3 years, a total of 7 outbreaks with 195 hospitalized people, 19 HUS cases, in which 2 people died (CDC, 2011 (b)), were recorded.

Practically in the entire world, including in many European countries, sporadic cases of *E. coli* O157:H7 outbreaks have been identified. However, they are less reported and investigated than in the USA, probably because the European Centre for Disease Prevention and Control (ECDC) was only established in 2005 (ECDC, 2012). By this reason, it's more difficult to estimate, before that year, the real number of cases and their focus diseases (Doyle *et al*., 2006).

The major *E. coli* O157:H7 outbreak, since this strain was first discovered in 1982, happened in Sakai, Japan in 1996, involving a total of 12,680 infected cases and 12 deaths, due to the consumption of salads in children's school (Doyle *et al*., 2006; FPJ, 2011)

In Europe, the biggest EHEC outbreak happened recently, in 2011, and was caused by a mutant strain, *E. coli* O104:H4, being reported more than 3,000 cases of illness and more than 50 deaths in 15 countries of the European Union (WHO/Europe, 2011). The biggest outbreak caused by *E. coli* O157:H7 reported so far, occurred in Scotland during 1995, with 633 recorded infection cases and with drinking water supply established as the focus infection (Doyle *et al*., 2006; FPJ, 2011)

Table 1 shows a compilation of the 20 biggest *E. coli* O157:H7 worldwide outbreaks recorded between 1982 and 2011, as well as the corresponding focus infection.

Table 1: Compilation of the 20 biggest *E. coli* O157:H7 worldwide outbreaks recorded from 1982 to 2011. [Adapted from: Doyle *et al*., 2006; CDC, 2011(b)]

Year	Cases Number	Local; Focus Infection
1996	12,680	Sakai, Japan; Salad
2000	2,300	Canada, Water
1999	>1000	EUA; Water
2000	788	EUA; Ground meat with cross-contamination
1992-93	>700	EUA; Hamburgers in fast-food restaurant
1995	633	Scotland; Water
1991	521	Canada; Ground meat
1996-97	512	Scotland; Meat
1996	503	Scotland; Lunch food
1997	332	United Kingdom; Fast-food restaurant
1999	329	EUA; Meat
1990	243	EUA; Water
2006	199	EUA; Fresh spinach
1999	159	Canada; Petting Zoo
2005	157	United Kingdom; Undercooked Meat
1998	157	EUA; Water
1999	143	Canada; Salame
1999	127	EUA; Water
2005	120	Sweden; Lettuce
1999	114	United Kingdom; Milk

1.1.3. E. COLI O157:H7 VIRULENCE FACTORS

The pathogenicity of EHEC bacteria is associated with a number of factors, including the production of various cytotoxins, designated by Verotoxin 1 and 2 (VTECs) in UK, or Shiga-like toxins Stx1 and Stx2 (STECs) in the United States.

Shiga-like name derives from the fact, that these toxins are similar to those produced by *Shigella dysenteriae* type I. Nevertheless, to cause disease, these toxins have to be associated with the intimin protein, encoded by *eae* gene (*E. coli* attaching and effacing), that enables the connection with the intestinal wall cell of the host (Feng, 1996; Tokarskyy *et al*, 2008). There are other accessory virulence factors, such as plasmid pO157 that encodes the following proteins: *espP* (extracellular serine protease), *katP* (katalase-peroxidase), *hlyA* (hemolysin A) and *etp* (*E. coli* tyrosine-protein) (Kaper *et al*., 1998; Bastos, 2009).

The chromosomal region that encodes all the pathogenic virulence factors is known as the *Locus of Enterocyte Effacement* (LEE) and has about 35 Kb (Elliott *et al.,* 1998), represented on Figure 1.

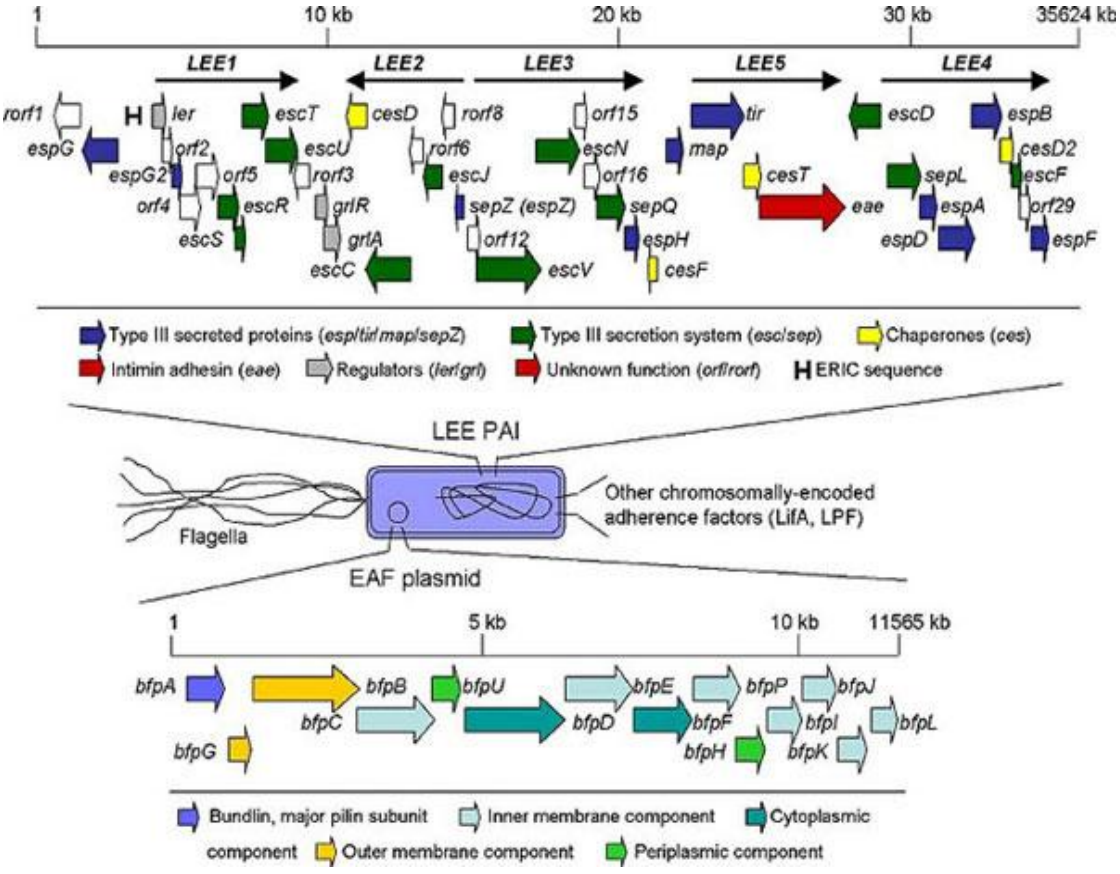

Figure 1: Pathogenic chromosomal LEE of *E. coli* O157:H7 and their genes. [Adapted from: Torres, 2009]

LEE encodes the type III secretion system and all proteins associated with the formation of pathological lesions in the intestinal epithelial cells, known as *Attaching and Effacing* (A/E) mechanism (McDaniel *et al*., 1995; Kaper, *et al*., 1998; Bastos, 2009). The ability to produce lesions A/E type required intimin protein, 94kDa, responsible for joining the bacterium to the enterocytes (epithelial cells of intestine),

disruption of microvilli and cytoskeleton of the host cell, resulting in the production of a pedestal base, rich in polymerized actin (McDaniel *et al.*, 1995; Kaper, *et al.*, 1998; Jay, 2005).

Due to the specificity of LEE region to encode the pathogenic virulence factors for *E. coli* O157:H7, the selection of DNA Probe and Target sequences were chosen in this region, more specifically in the intimin encoding gene, *eae*. The method used for the selection of these oligonucleotides is described in the following *Chapter II- Materials and Methods*.

1.1.4. METHODS FOR E. COLI O157:H7 DETECTION

A variety of conventional methods have been proposed to detect and screen many microorganisms, including *E. coli* O157:H7, such as the following ones (Wu *et al.*, 2004; Velusamy *et al.*, 2010):

- ***Culture and colony counting methods***: these remain as the most reliable and accurate techniques for foodborne pathogen detection. Nevertheless, they require up to 7 days to yield results, as they rely on the ability of microorganisms to multiply into visible colonies;
- ***Immunology-based methods***: immunological detection with antibodies is perhaps the only technology that has been successfully employed for the detection of bacterial cells, spores, viruses and toxins alike. The problems that may arise with immunoassay-based methods are the low sensitivity of the assays, low affinity of the antibody to the pathogen or other analyte being measured, and potential interference from contaminants (Meng and Doyle, 2002);
- ***Polymerase Chain Reaction (PCR)***: in theory, this method, can detect a single copy of a target DNA sequence, and thus, can be used to detect a single pathogenic bacterium in food. However, when testing food samples, false negative PCR results can occur and may be due to interference with target-cell lysis necessary for nucleic acid extraction, nucleic acid degradation and/or direct inhibition of the PCR enzyme. Therefore, it is essential to include appropriate controls for the application of PCR to the detection of pathogens in food samples.

Since all the traditional methods to detect foodborne pathogens are often time-consuming, there is a need for new technologies that have to be rapid, reliable, simple, specific and sensitive (Meng *et al*., 2001; Velusamy *et al*., 2010). In addition, the majority of conventional methods require intricate specific instrumentation, highly trained operators and cannot be used *in situ* at low cost (Heo *et al*., 2009; Velusamy *et al*., 2010)

Recently, modern biological techniques have been proposed for detection of pathogenic microorganisms (Wu *et al*., 2007), such as:

- ***Real-time PCR****:* allows detection, amplification and quantification of DNA in real time (Yoshitomi *et al*., 2003; Fu *et al*., 2005);
- ***Nanoparticles****:* used for detection of various biological molecules including proteins, enzymes, DNA, antigens and antibodies (Tiwari *et al*., 2011);
- ***Biosensors****:* used to detect biological or chemical complexes in the form of antigen-antibody, nucleic acids, enzyme-substrate or receptor-ligand compounds.

Much of this new biological techniques research activity has been made in biosensors area. An overview of recent efforts using biosensors in the field of food pathogen detection will be detailed in the following section.

2. BIOSENSORS

A biosensor is, in general, an analytical device, which converts a biological response into an electrical signal. It consists of two main components: a bioreceptor or biorecognition element, which recognizes the target analyte and a transducer, for converting the recognition event into a measurable electrical signal (Healy *et al.*, 2007; Wu *et al.*, 2007, Velusamy *et al.*, 2010).

Figure 2 shows schematic diagram of a biosensor.

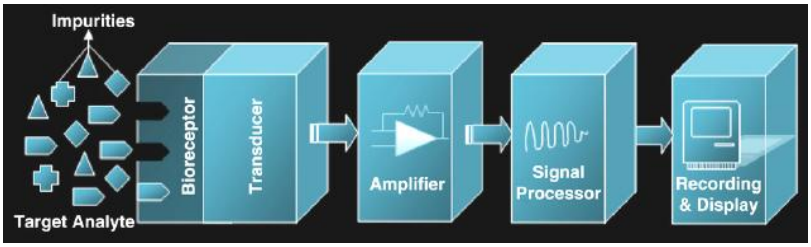

Figure 2: Schematic diagram of a biosensor. [Adapted from: Velusamy *et al.*, 2010]

Velusamy *et al.*, 2010, explains that: "the bioreceptor recognizes the target analyte and the corresponding biological responses are then converted into equivalent electrical signals by the transducer. The amplifier in the biosensor responds to a small input signal from the transducer and delivers a large output signal that contains the essential waveform features of an input signal. The amplified signal is then processed by the signal processor, where it can later be stored, displayed and analysed".

In the past years, biosensors have been applied to a variety of analytical problems in different areas, such as in medicine, environment, food process industries, security and defence.

Biosensors can be classified accordingly to their bioreceptor or their transducer type (Vieira, 2007; Velusamy *et al.*, 2010; Arora *et al.*, 2011), as shown in Figure 3.

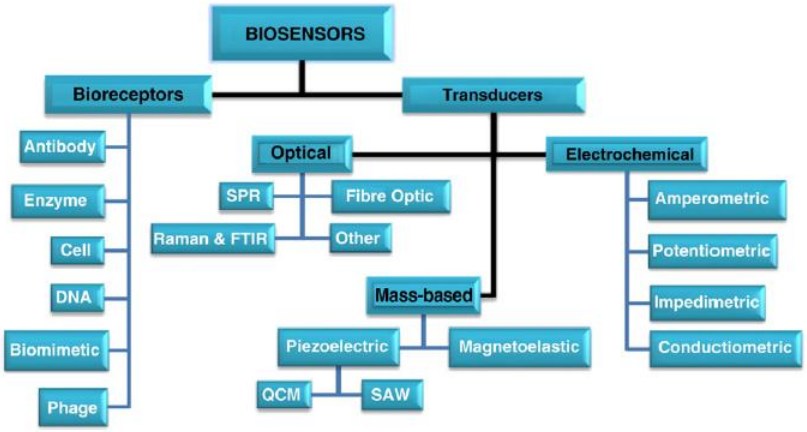

Figure 3: Classification of a biosensor. [Adapted from: Velusamy *et al.*, 2010]

Based on the bioreceptor type, they can generally be classified into six different major categories (Velusamy *et al.,* 2010):

- ***Antibody/antigen:*** are based on the lock and key fit (Vo-Dinh and Cullum, 2000), where an antigen-specific antibody fits its unique antigen in a highly specific manner. The antibody may be polyclonal, monoclonal or recombinant, depending on their selective properties and the way they are synthesized;
- ***Enzymes:*** are chosen based or their specific binding capacity and their catalytic activity (Vo-Dinh and Cullum, 2000). Enzymes offer the advantages of high sensitivity, possibility of direct visualization of results and being stable for years, when the proper conditions are maintained. Nevertheless, they present disadvantages, such as the necessity of multiple assay steps and the possibility of interference with endogenous enzymes;
- ***Nucleic acids/DNA:*** are based on the fact that each organism has a unique DNA sequence, any self-replicating microorganism can be easily identified by using nucleic acids as a target analyte and their matching complementary base pairs. Biosensors based on nucleic acids as a biorecognition element are simple, rapid, and inexpensive and hence they are widely used for pathogen detection. However, they present the disadvantage of easy DNA damage by physical, chemical and biological activities that may cause irreversible damage and changing the structural of DNA and the base sequence, which in turn, disturbs the DNA hybridization and the detection of pathogen;
- ***Cellular structures/cells:*** are based on whole cell/microorganism or a specific cellular component that is capable of specific binding to certain species;
- ***Biomimetic:*** a receptor that is fabricated and designed to mimic a bioreceptor (antibody, enzyme, cell or nucleic acid) is often termed a biomimetic receptor. Molecular imprinting technique is one of the techniques used to produce artificial recognition sites by forming a polymer around a molecule which can be used as a template. Nevertheless, only very few applications have been successfully reported, so far, for foodborne pathogen detection;
- ***Bacteriophage:*** recently, bacteriophages have been employed as biorecognition elements for the identification of various pathogenic microorganisms. Bacteriophages are viruses that bind to specific receptors on the bacterial surface, in order to inject their genetic material inside the bacteria. Since the

recognition is highly specific, it can be used for typing bacteria and hence opened the path for the development of specific pathogen detection technologies.

The transducer plays an important role in the detection process of a biosensor. As referred before, biosensors can also be classified based on the transduction methods they employed (Velusamy *et al.,* 2010):

- ***Optical-based biosensors:*** have received considerable interest for bacterial pathogen detection due to their sensitivity and selectivity. They are based on absorption, reflection, refraction, dispersion, infrared, Raman, chemiluminescence, fluorescence and phosphorescence detection. Being the most commonly employed techniques of optical detection the Surface Plasmon Resonance and fluorescence, due to their sensitivity;
- ***Electrochemical biosensors:*** can be classified into amperometric, potentiometric, impedimetric and conductometric, based on the observed parameters such as electric current, electric potential, electric impedance and electric conductance, respectively. Although the electrochemical detection has several advantages, like low cost, ability to work with turbid samples and easy miniaturization, their sensitivity is slightly limited when compared to optical detection;
- ***Mass-sensitive biosensors:*** the transduction is based on small changes in mass, being suitable for very sensitive detection. The principal means of mass analysis depends on the use of piezoelectric crystals, which can be made to vibrate at a specific frequency with the application of an electrical signal of a determined frequency. The two main types of mass based sensors are the Quartz Crystal Microbalance (QCM) and Surface Acoustic Wave (SAW).

2.1. BIOSENSORS TO DETECT *E. COLI* O157:H7

A quick search on the scientific database *ScienceDirect* using the keywords: *Biosensor* and *E. coli O157:H7*, results in more than 477 scientific papers between 1993 and 2012. Restricting this search for the year of 2011 and beginning of 2012, we can

account 100 papers, fact which shows the importance and interest of using this biological technique for the detection of this pathogen.

Table 2 shows some published papers, evidencing the great diversity of transducers used in biosensors when applied to the detection of *E. coli* O157:H7.

Table 2: Examples of scientific papers published concerning biosensors development for *E. coli* O157:H7 detection.

Transducer	Mode detection	Limit detection	Assay time	References
Electrochemical	Potentiometric	$7.1x10^2$ cells/ml	45 min	Gehring *et al.*, 1998
Optical	Chemiluminescence enzyme immunoassay	10^1 to 10^2 cells/g	24 h	Kovacs and Rasky, 2001
Electrochemical	Amperometric	$6x10^2$cells/ml	2 h	Ruan *et al.*, 2002
Electrochemical	Amperometric	81 CFU/ml	6 min	Muhammad-Tair and Alocija, 2004
Electrochemical	Amperometric	$1.6x10^1$-$7.2x10^7$ CFU/ml	15 min	Varshney *et al*., 2005
Mass-sensitive	Quartz Crystal Microbalance	$2.67x10^2$-$2.67x10^6$ CFU/ml	30 min	Mao *et al*., 2006
Mass-sensitive	Quartz Crystal Microbalance	10^6 CFU/ml	Not reported	Wu *et al*., 2007
Optical	Bacteriophage-based bioluminescene	10CFU/ml	4 h	Brigati *et al.*, 2007
Optical	Fluorescence microscopy	10^2 CFU/ml	Not reported	Choi and Oh, 2008
Electrochemical	Impedimetric	$2.5x10^4$-$2.5x10^7$ CFU/ml	3 h	Dweik et al., 2012

The following subchapter will reveal the particularities of DNA bioreceptores (Genosensors) and mass-sensitive transducers (QCM-D), used in the present work.

2.2. DNA AND GENOSENSOR

There are two types of nucleic acids, RNA (ribonucleic acid) and DNA (deoxyribonucleic acid) which form the basis of biochemical hereditary, present in all living organisms, with the exception of virus, which have only one of them.

Located mainly in the nucleus of all eukaryotic cells, these chemical polymers contain the genetic instructions responsible for the development and functioning of living organisms.

The DNA structure as we know today was proposed in 1953 by James D. Watson and Francis H.C. Crick, with the invaluable collaboration of the Rosalind Franklin X-ray crystallographer. They proposed that the DNA was a three dimensional helix structure formed by two antiparallel strands of repetitive building blocks, called nucleotides, linked by hydrogen bonds (Watson and Crick, 1953; Nobel Media, 2011). Each nucleotide consists of a ribose sugar and phosphate, which represent the strand backbone, and one of four different nitrogenous base units. These bases are *adenine* (A), *guanine* (G), *thymine* (T) and *cytosine* (C), and based on the chemical structure, they can be classified into *purines* (double heterocyclic rings) including A and G bases, and *pyrimidine* (single heterocyclic ring) including T and C bases. The double-stranded DNA (*ds*DNA) is formed via hydrogen bonds between two bases, located in each one of the double strands, where *adenine* always combines with *thymine* and *guanine* with *cytosine*. This phenomenon is referred as *hybridization*.

The most important function of DNA is the production of proteins through a cycle discovered known as the central dogma of molecular biology. In this process, segments of DNA are transcribed to messenger ribonucleic acid (*m*RNA) via certain enzymes, and the *m*RNA is later translated into proteins. RNA is similar to DNA except it is single stranded and the *thymine* (T) bases are replaced by *uracil* (U) bases and a ribose on the nucleic (Nasef, 2010).

Based on the exceptional specificity that arises from the inherent ability of complementary DNA bases to hybridize, the use of selected DNA probes provides an extremely useful, simple, quick and inexpensive tool to detect complementary DNA sequences in a sample.

By this way, genosensors have become increasingly prominent in the literature because of the opportunities they offer for better diagnosis, prevention and treatment of many human diseases (Abu-Salah *et al*., 2010).

These technologies are based on DNA immobilization of a sequence in different physico-chemical transducers that convert the hybridization in an electrical or optical signal (Vieira, 2007; Wu *et al*., 2007), as shown in Figure 4.

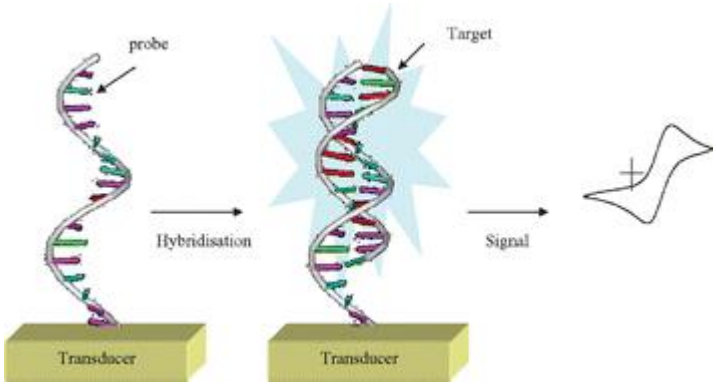

Figure 4: Schematic representation of obtaining an electrical signal with a biosensor based on DNA. [Adapted from: RSC, 2012]

In DNA hybridization, the target gene sequence is identified by a DNA probe, that can form a double-stranded hybrid with its complementary nucleic acid with high efficiency, in the presence of a mixture of many different non-complementary nucleic acids (Abu-Salah *et al*., 2010).

The standard method for detecting nucleic acid hybrids is by labelling the probe with radioactive nucleotides, chemiluminescent dye or various hapten molecules, such as biotin (Wu *et al*., 2007), in order to obtain an amplified signal.

2.2.1. Immobilization of DNA Probes and SAM Formation

The idea of immobilization and hybridization of DNA molecules to a solid support was first described by Denhardt (1966), which led to the development of new methods for identifying sequences in the genomic DNA and cloning (Denhardt, 1966; Pedroso, 2011).

One of the key processes for the development of a genosensor is the immobilization of short DNA probes or oligonucleotides, usually with 20 nitrogenous bases, on the electrode surface/transducer.

Immobilization not only guarantees a close contact between the bioreceptor and the transducer but also helps the stabilization of the biological structure, reinforcing its functional and storage durability. Several methods can be used to immobilize the DNA probe on the sensors surface, such as, adsorption, covalent binding, affinity or embedding in polymers (Berganza *et al*., 2007).

The direct adsorption of single-stranded DNA (*ss*DNA) probe on electrode surface by Self-Assembled Monolayer (SAM) has been described as one of the immobilization methods with more stability, simplicity, efficiency, arranged immobilization and low cost.

SAMs are crystalline chemisorbed organic single layers formed on a solid substrate by spontaneous organization of molecules (Chechick and Stiring, 1999).

Another advantage for the use of SAMs is the prevention of the non-specific adsorption of DNA on the electrode surface during the immobilization and hybridization procedures (Freire *et al*., 2003; Berganza *et al*., 2007).

The vertical spontaneous organization that is obtained by SAM formation with DNA probes, not only enables a higher surface area available for immobilization, but also helps in hybridization of DNA target.

One of the most important classes of SAMs is based on the strong adsorption of sulphur-based compounds (thiols, disulphides, sulphide) and related moieties on coinage metals, particularly Au, Ag, Cu, as well as, Pt, Hg, Ga, As and InP surfaces (Prashar, 2012).

During the formation of SAMs, adsorption is generally carried out by placing the substrate in dilute solution of thiols. Any solvent which is capable of dissolving the thiol can be used. Adsorption can be also carried out under potential control or from the vapour phase (Finklea, 1996).

Gold metal (Au) has been the most well studied surface for the self-assembly process by using alkanethiols. In the literature, Au is described as the surface with the best characteristics for forming alkanethiol SAM (Su *et al*., 2004; Berganza *et al*., 2007; Tappura *et al*., 2007; Carrara *et al*., 2010; Ansorena *et al*., 2011).

The first method using a post-processing of DNA thiol probes was reported by Herne and Tarlov (1997), where Au electrodes were initially incubated with DNA probes, and then exposed to an alkanethiol solution, 6-mercapto-1-hexanol (MCH). They characterised this phenomena by employing X-ray photoelectron spectroscopy

(XPS), ellipsometry, P-radiolabering, neutron reflectivity and electrochemical methods (Herne and Tarlov, 1997; Nasef, 2010).

This work was based on the fact that DNA thiolated probes could interact non-specifically with gold electrode surfaces through DNA nitrogen-containing bases and not through the S-Au bond, which affects the accessibility of the immobilised probes to hybridize successfully with the DNA targets in the sample solutions (Nasef, 2010). By this way, MCH solution worked as a blocking agent on DNA probes, and also prevented the non-specific adsorption of DNA on the electrode surface during the immobilization and hybridization procedures (Berganza *et al.*, 2007; Lucarelli *et al.*, 2008, Keighley *et al.*, 2008; Carrara *et al*., 2010), represented in Figure 5.

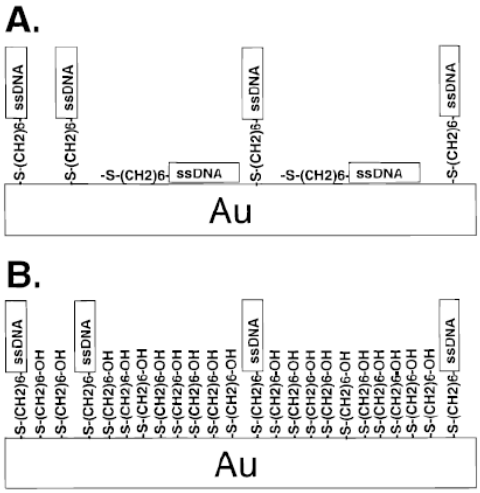

Figure 5: Schematic representation of SAMs formation on gold surface (Au). A) thiolated DNA probes; B) MCH. [Adapted from: Nasef, 2010]

More recently, other works have described successful SAM formation using other blocking agents, such as groups derived from ethylene-glycol and lipoate (Tappura *et al*., 2007; Carrara *et al*.,2010), by direct addition of MCH mixed with thiol DNA probes (co-immobilization), or even, with -COOH terminations (Nakamura *et al*., 2006).

2.2.2. DETECTION OF IMMOBILIZATION AND HYBRIDIZATION SIGNAL

Surface and interfacial study process is extremely important for the choice of the immobilization method on the electrode surface and the detection of associated problems with this step. Thus, the study of these processes assists in choosing the best immobilization system, as well as, the advantages and disadvantages of each method (Damos *et al*., 2004).

Generally, to obtain the maximum signal in the biosensor is also necessary to have maximum concentrations of immobilized and hybridized oligonucleotides. According to Steel and co-workers (1998), the signal obtained will increase until the maximum value of $4x10^{12}$ probes/cm^2, after this saturation value, the amount of hybridized target quickly decreases, as well as, the signal recorded in the biosensor (Steel *et al*., 1998; Keighley *et al*., 2007).

Therefore, techniques capable of assessing surface and interface phenomena are of great importance in the development of new biosensors. The following techniques are among the most used (Pedroso, 2011):

- **Surface Plasmon Resonance (SPR):** uses an optical method to measure the refractive index near (within ~300 nm) a sensor surface;
- **Electrochemical Impedance Spectroscopy (EIS):** usually measured by applying an AC (alternating current) potential difference to an electrochemical cell and then measuring the current through the cell;
- **Quartz Crystal Microbalance (QCM)**: piezoelectric mass-sensing devices based on quartz crystal resonance frequency.

3. QUARTZ CRYSTAL MICROBALANCE (QCM)

The QCM is a very sensitive sensor that detects mass changes based on the piezoelectric effect, which was discovered in 1880, by the Curie brothers (Höök and Rudh, 2005). This effect is defined as the property of certain materials to develop a potential difference between their deformed surfaces, which is proportional to the applied pressure.

One of the materials that shows this property are the piezoelectric crystals, (Yamasaki, 2005) being *α-quartz*, the crystalline form of silicon dioxide (SiO_2), the most commonly used (Höök, 2004).

In a quartz crystal, the electric field is applied over the metallic electrodes on both sides of the disk, usually Au, but also can be substituted by platinum (Pl), silver (Ag), among others.

3.1. PRINCIPLE OF QCM DEVICE

The basic principle of QCM is that, a tiny mass change can cause inertial changes on the crystal surface that, subsequently lead to oscillation frequency shifts on the crystal.

In 1959, Sauerbrey established the linear relationship between the mass changes at the QCM electrode surface and the oscillation frequency changes in the quartz crystal, nowadays being designated as the Sauerbrey equation (Chang *et al.*, 2008), and shown as Equation 1:

$$\Delta f = -\frac{2f_0^2 \Delta m}{A\sqrt{\rho_q \mu_q}} \qquad [1]$$

where,

Δf, frequency change (Hz);

f_0, crystal piezoelectric oscillation frequency (Hz)

A, piezoelectric active crystal area (m^2)

Δm, mass change on the metallic material surface (g)

ρ_q, density of quartz (ρ_q=2.648 g/cm^3)

μ_q, shear modulus of quartz for AT-cut crystal (μ_q=2.947x10^{11} $g/cm.s^2$)

This relationship, later demonstrated the extremely sensitive nature of QCM toward mass changes on its surface. Its accuracy is so high that is possible to detect frequency differences smaller than 1Hz that corresponds to a mass difference of a few ng/cm^2 (Höök, 2004; Chang *et al*., 2008).

Using the QCM device and the Sauerbrey equation it is possible to detect in real-time, the increase of mass bound to the quartz crystal surface, that causes a decrease of oscillation frequency, or the decrease of mass bound into the quartz crystal, that causes an increase on the oscillation frequency.

Nevertheless, for many years, QCM devices were regarded as just gas-phase detectors, until the 1980s, when Nomura and Okuhara designed oscillator circuits that could apply this sensor not only in vacuum or air, but also in liquids. This created the opportunity of using this equipment as a useful tool in the field of biotechnology and biosensors (Liu *et al*., 2010).

The QCM device, due to its simplicity and operation cost effectiveness, has been extensively investigated as a transducer for the detection of gene mutations, genetically modified organisms, foodborne pathogens and toxicology, in hybridization based DNA sensors (Mao *et al*., 2006; Pedroso, 2011). Other advantages for the use of QCM are the possibility of obtaining results in real-time, its high sensitivity and reliability (Lucarelli *et al*., 2008).

3.2. QCM WITH DISSIPATION FACTOR (QCM-D)

QCM-D is a special QCM device for surface analysis developed and patented by *Q-Sense*® Company.

This device allows the discrimination of frequency *f*, and dissipation factor *D*.

Figure 6 shows a typical QCM-D with one chamber, *Q-Sense E1* that was used in the present work.

Figure 6: QCM-D device with one chamber - Q-Sense E1. Adapted from: Q-Sense, 2012(b)

Table 3 shows the sensors, sample handling system, frequency and dissipation characteristics, for *Q-Sense E1*.

Table 3: Characteristics of the sensors, sample handling system, frequency and dissipation for Q-Sense E1. [Adapted from: Q-Sense, 2012(b)]

Sensors and sample handling system	Characteristics
Number of sensors	1
Volume above each sensor	~ 40µl
Minimum sample volume	~ 300µl
Working temperature	18 to 45ºC controlled via software stability ±0.02K
Flow rates	0-1 ml/min
Sensor crystals	5MHz, 14mm diameter, polished, AT-cut, gold electrodes
Frequency range	1-70MHz (up to the 13th overtone, 65MHz for a 5MHz crystal)
Maximum time resolution, 1 frequency	Up to 200 data points per second
Maximum mass sensitivity in liquid	~ 0.5 ng/cm^2
Normal mass sensitivity in liquid	~ 1.8 ng/cm^2
Maximum dissipation sensitivity in liquid	~ 0.04x10^{-6}
Normal dissipation sensitivity in liquid	~ 0.1x10^{-6}
Typical noise peak to peak (RMS) in liquid	~ 0.16Hz (0.04Hz)

The dissipation factor, allows to the operator information about the oscillation in the layers, such as, the adhesion/binding phenomena of the quartz crystal electrode (Chang, 2008), as well as, the viscoelastic properties in the interface (Lucarelli *et al*., 2008). This sensor's dissipation energy is measured by recording the response of a freely oscillating sensor that has been vibrated at its resonance frequency.

D is defined as shown in Equation 2 (Q-Sense, 2012(a)):

$$D = \frac{E_{\text{lost}}}{2\pi E_{\text{stored}}} \qquad [2]$$

where,

E_{lost} = Energy dissipated during one oscillation cycle

E_{stored}= Total energy stored in the oscillator

By measuring the dissipation, it is possible to determinate if a soft film (water rich) or a rigid film (water poor) has been formed on the surface. Only when the film is fairly rigid the Sauerbrey equation gives a good estimation of the adsorbed mass. When the dissipation value typically reaches above 1×10^{-6} per 10 Hz, the film is too soft to function as a fully coupled. This means that the Sauerbrey equation, which is normally used to calculate the mass directly from the change in frequency, will underestimate the mass. However, by measuring both dissipation and frequency, at several harmonics with QCM-D, it is possible to extract the correct thickness estimation even in these cases. This also makes it possible to calculate the viscoelastic and structural properties using a viscoelastic model (Voight model) incorporated in the *Q-Sense* software, the *Q-Tools* (Q-Sense, 2012(b)). Therefore, the adhering film can be characterized in detail by viscosity and elasticity, as the correct thickness that may be extracted even for soft films, when certain assumptions are made (Chang, 2008).

Measurements can be carried out on any surface that can be applied as a thin film, including polymers, metals and chemically modified surfaces.

The common QCM-D study areas are Drug development, Environmental, Energy, Cell and Molecular biology, Surfactants, Biomaterials, Polymers and Biosensors (Q-Sense, 2012(a)).

3.2.1. DATA ANALYSIS BY Q-TOOLS SOFTWARE

Generally, directly connected to the oscillator circuit there is a frequency counter, which is responsible for monitoring the frequency and dissipation variations of the crystal oscillation. Moreover, both are connected to a computer to obtain, collect and process the data information, as shown in Figure 7.

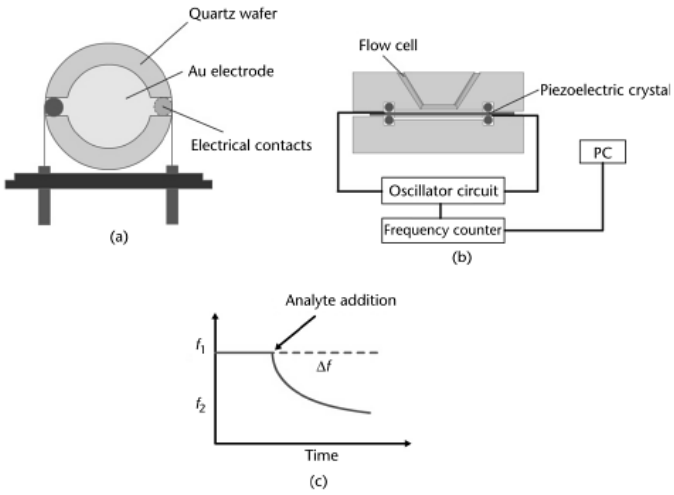

Figure 7: Scheme of a typical QCM device: (a) Quartz crystal electrode; (b) Flow cell scheme in liquid media, including the oscillator circuit and frequency counter; (c) Mass bound and frequency change into QCM. [Adapted from: Liu *et al*., 2010]

Following to the real-time data recording from *Q-Sense* software, the data information can be directly processed in the *Q-Tools* software, by choosing the Sauerbrey equation in one specific harmonic (normally the 9th overtone) and obtaining the parameters in study, such as, mass changes, thickness and/or density.

The overtone mode is usually employed to increase the sensitivity (Yoshimoto *et al.*, 2004), and in QCM-D device, the overtones goes from n=1 until n=13. Only odd harmonics can be excited electrically because only these induce charges of opposite sign at the two crystal surfaces (Goka *et al.*, 2000). However, in QCM-D with fundamental frequency of 5MHz, the best agreement between theory and experiment is reached with planar, optically polished crystals for overtone of n = 9. On low harmonics, energy trapping is insufficient, while on high harmonics, interfere with the main resonance (*Goka et al.*, 2000).

3.3. DNA *E. COLI* O157:H7 DETECTION STUDIES WITH QCM- D

The use of QCM technique to check the DNA immobilization and sequence-specific DNA hybridization was first used in 1998, by Prof. Yoshio Okahata and co-workers at the Tokyo Institute of Technology in Japan, and later extended by the same group to studies of template-directed DNA synthesis (Höök and Rudh, 2005). However, it was just in 2006, that the first scientific paper reporting its application for the rapid detection of *E. coli* O157:H7 was published, characterized by means of QCM and using streptavidin-coated ferrofluid nanoparticles as "mass enhancers". In this study, this

research group demonstrated the effectively amplified signals in frequency shifts using nanoparticles, resulting in an improved detection limit of 10^{-12} M for synthesized oligonucleotides, which were about four orders lower than the same assays performed without nanoparticle amplification (Mao *et al*., 2006).

In 2007, Wu and co-workers developed a DNA piezoelectric biosensing method for real-time detection of *E. coli* O157:H7 in a circulating-flow system. In this study, a spacer (12-dT) linked to the probes enhanced the detection signals and reduced the spheric interference of the support on the hybridization behaviour of the immobilized oligonucleotides. In this study, it was also found that thiol modification on the 5'-end of the probes was essential for probe immobilization on the gold surface of the QCM device.

In 2009, Poitras and Tufenkji reported the development of an immunosensor for detection of *E. coli* O157:H7 using for the first time, a QCM-D device to study this strain. In this study the application of the initial D_{slope} as a useful sensor response in QCM-D technology was reported. The authors concluded that D_{slope} provides an important advantage for rapid bacteria detection, and also that the slope in the *D* shift can be a useful signal to be monitored in a broad range of QCM-D applications (Poitras and Tufenkji, 2009).

Exploring scientific databases, such as *ScienceDirect* and *PubMed* it can be determined that the great majority of studies for detecting *E. coli* O157:H7, using the QCM device, were made for the development of immunosensors. In none of these databases was found a study published until 2011, with the purpose to develop a genosensor of *E. coli* O157:H7 characterized by means of QCM-D device and confirmed by epifluorescence microscope, as the present work purposes.

4. EPIFLUORESCENCE MICROSCOPE

The fluorescence microscope was devised in the early part of the twentieth century by August Köhler, Carl Reichert and Heinrich Lehmann, among others. However, the potential of this instrument was not realized for several decades, until the 1990s, after the development of new techniques in different areas, such as, molecular biology, cellular biology and biotechnology (Olympus, 2012).

The causes for the advancement of fluorescence microscope were the high sensitivity, selectivity, almost non-invasive to the biological systems and the easily obtained imaging. The use of this technique allows, the observation of biological and biochemical reactions, such as, pH changes, biomass immobilized in real-time and other biological events intended to be observed (Slavik, 1997; Junior, 2008).

The fluorescence phenomenon is a type of luminescence (light emission), characterized by energy absorption and reemission in a form of light. Depending on the source of energy, the luminescence can be classified as: electroluminescence, radioluminescence, chemiluminescence and photoluminescence.

Photoluminescence occurs when electrons from a fluorochrome absorb photons with *hv* energy from a source (lamp or laser beam) and pass from a fundamental (low energy) to an excited state (higher energy). When the electrons return to the fundamental state, this difference in energy is emitted in the form of a visible photon. Therefore, the fluorescence microscopy technique consists in capturing the released photons, usually, in the visible spectrum and with a longer wavelength than the excitation light, generating an image. The disadvantage of this technique is the over time degradation of the fluorochrome, known as photobleaching, where the fluorochromes lose its light signal intensity by the interaction with oxygen molecules (Junior, 2008; Metz, 2011).

Currently, most of the fluorescence microscopes use epifluorescence, that is a type of excitation-emission configuration, in which both illumination and emission light travel through the objective (Harris *et al*., 2005, Alves, 2009).

The principle basis is that the epifluorescence microscope needs to filter the radiation so that only the desired wavelength would be retained. This succeeding radiation will then collide with specimen atoms and get excited to a higher level of energy. When they return at a lower energy level, they are able to emit light. In order to become visible through the emitted light, the microscope uses a second filter. In this area, the lower energy of emitted light with a longer wavelength is used and fluorescing

areas are observed with a high contrast against the dark background on the microscope (Harris *et al*., 2005).

Figure 8 shows a scheme of a typical Epifluorescence microscope and its components.

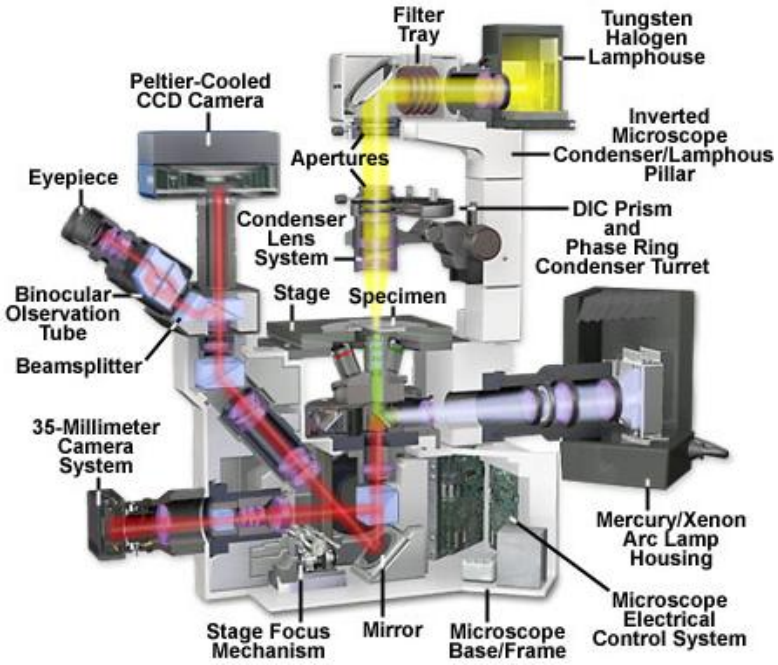

Figure 8: Scheme of an Epifluorescence microscope. [Adapted from: Olympus, 2012]

Because of the key features of an Epifluorescence microscope, it is now becoming extremely used in the biological and medical fields.

4.1. FLUORESCENCE AND FLUOROCHROMES

As mentioned above, the use of fluorescence microscope requires the presence of fluorescent molecules, which may be natural or added to the biological sample under study. The fluorochromes, also known as, fluorophores or fluorescent dyes, are molecules capable of emitting fluorescent light. In reality, only a portion of these molecules has the property of emitting fluorescence, and this portion is named fluorochrome. In general, these groups are aromatic rings or highly conjugated systems which absorb a wide band of wavelengths (Junior, 2008), and are specifically conjugated to a part of the sample depending on what the observer wishes to study.

The fluorochromes and its characteristics, used in the present work are described in the following *Chapter III- Materials and Methods,* as well as, the specific used filters in the epifluorescence microscope.

CHAPTER III - MATERIALS & METHODS

1. SELECTION AND MODIFICATION OF OLIGONUCLEOTIDES

One of the most important pre-analytical steps to the development of a genosensor is the choice of a genomic location for the specific recognition of an organism.

Currently, the design of the oligonucleotides has the advantage of decades in experience and development of new biological techniques, such as, molecular and cell biology, which allows the access to numerous bioinformatics tools. Combined to these tools, the access to genomic databases which contain information of almost all organisms, allows the access to genomic sequences information and/or a choice of a specific gene. With this genomic information and using specific bioinformatics tools, it is possible to design specific primers for PCR, choose restriction enzymes or check alignments with homologous sequences, in this last case by using BLAST (*Basic Local Alignment Search Tool*).

After the selection and design of a specific recognition sequence, the DNA probe and target are usually obtained by PCR. This technique consists in a general way, in the reproduction *in vitro* of thousands of copies of a DNA fragment by thermocycles of denaturation, primer annealing and extension steps, in a few hours. Because it is a very sensitive and automatic technique, several problems and errors can occur, such as, external DNA contamination and its amplification, hairpins (secondary structures), polymerase errors, non-specific priming and nucleotide mutations on the amplified fragments.

1.1 DESIGN OF OLIGONUCLEOTIDES FOR *E. COLI* O157:H7 DETECTION

For this work, two complementary oligonucleotides were selected with 21-mer from *eae* gene of *E. coli* O157:H7. This gene encodes intimin adhesion protein, essential to occur the A/E mechanism and virulence.

Figure 9 shows a scheme representation of the *eae* gene of *E. coli* O157:H7 (EDL933 strain) obtained on NCBI (*Nacional Center for Biotechnology Information*).

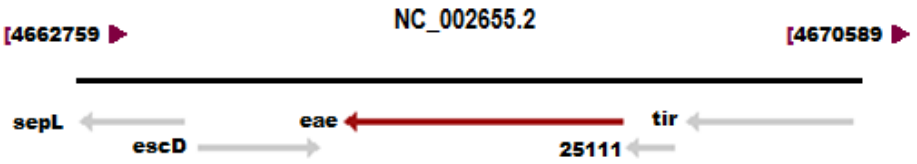

Figure 9: Scheme representation of the *eae* gene of *E. coli* O157:H7 obtained on NCBI, showing the size, ORF, local and code of this gene. [Adapted from: NCBI, 2012 (a)]

The specificity of the selected 21-mer sequence (DNA probe), to detect *E. coli* O157:H7 strain is an extremely important feature in a genosensor. In order to confirm the specificity and compatibility of the selected DNA probe sequence, a BLAST running on the NCBI was made, as shown in Figure 10.

```
Features flanking this part of subject sequence:
  91 bp at 5' side: LEE-encoded type III secretion system component
  154 bp at 3' side: intimin adherence protein

Score = 40.1 bits (20),  Expect = 0.093
Identities = 20/20 (100%), Gaps = 0/20 (0%)
Strand=Plus/Plus

Query  2        CAGCGTGGTTGGATCAACCT  21
                ||||||||||||||||||||
Sbjct  4655430  CAGCGTGGTTGGATCAACCT  4655449
```

Figure 10: Scheme of a BLAST on the NCBI to confirm the specificity and compatibility of the DNA probe of *E. coli* O157:H7. [Adapted from: NCBI, 2012(b)]

After selecting the Probe and Target sequences, they were acquired to *Sigma-Aldrich*®, with the following specifications:

- **DNA Probe**: a C6 alkanethiol (ThiC6) was added in the 5'-end, to facilitate the immobilization step. A fluorescence group, *Fluorescein* (FITC), was added in the 3'-end to identify the DNA probes in the immobilization step by Epifluorescence microscopy.
- **DNA Target**: a fluorescence group, *Texas red* (TxRd), was added in the 5'-end to identify the DNA target in the hybridization step by Epifluorescence microscopy.

The DNA Probe and Target sequences used in the present work are presented on Table 4.

Table 4: Representation of the DNA Probe and Target sequences with modifications on 5' and 3'-end.

Oligonucleotides	**5'-3' Sequence**
DNA Probe	5'- [ThiC6] ACAGCGTGGTTGGATCAACCT [FITC] -3'
DNA Target	5'- [TxRd] AGGTTGATCCAACCACGCTGT -3'

2. FUNCTIONAL DNA THIOL PROBES

The selected oligonucleotides were acquired in a lyophilized form, since this procedure enables the oligonucleotide conservation and stabilization for the transportation, at room temperature, and makes them less sensitive to nucleases degradation.

After delivery, oligonucleotides are typically dissolved in sterile water or buffer solution at a known concentration, divided into aliquots and stored at -20°C, for long time conservation, or at 4°C, for the using on a few days after.

In this specific work, because both requested oligonucleotides had modifications, *Sigma-Aldrich*® company, suggests that the oligonucleotides should be restored in 1x TE buffer solution (10mM Tris-CL+1mM EDTA, pH 7.5-8.0, (see *Appendix I*)), in order to maintain their stability.

In the case of the DNA Probe, which was modified with an added thiol group in the 5'-end, they were also supplied in a protected form, with the disulfide linkage intact to minimize the potential for oxidation, which can result in oligo dimer formation. Thereby, it was necessary to reduce the disulfide linkage with dithiothreitol (DTT) solution, so the thiol group could become free and functional.

The procedure recommended by *Sigma-Aldrich*®, for this type of modified oligonucleotides, is based on the chemical reaction presented in Equation 3, and includes two main steps, as following detailed in sections 2.1 and 2.2.

$$R\text{–}S\text{–}S\text{–}(CH2)6\text{–}Oligo + 0.1\ M\ DTT \rightarrow HS\text{–}(CH2)6\text{–}Oligo \quad [3]$$

2.1. SULFHYDRYL ACTIVATION BY REDUCING DISULFIDE LINKAGE WITH DTT

1. A 100 mM DTT (*Sigma-Aldrich, Nº. D9779*) solution in phosphate buffered saline (PBS, (*Sigma-Aldrich, Nº. P5368*)), pH 8.3-8.5, (see *Appendix II*), was prepared using the next rates:
    a. For 0-12.5 A_{260} units, the non-reduced oligonucleotides should be dissolved in 125 µl of 100 mM DTT;
    b. For more than 12 A_{260} units of oligonucleotides, a ratio 10:1 (µl DTT: A_{260} units) should be maintained;
    c. For 5 ml of the 100 mM DTT, added 77.13 mg of the DTT powder;

2. The DTT + oligonucleotides solution was incubated one hour, at room temperature and in the dark.

2.2. REMOVAL OF DTT AND OTHER REACTION BYPRODUTS

After the sulfhydryl activation by DTT solution, the DTT and other reaction byproducts must be removed to avoid interferences with subsequent conjugations.

Different procedures can be used to remove the excess of DTT and byproducts, such as desalting through gel filtration chromatography, by using NAP-10 or Sephadex column. Another procedure to remove DTT salts, consists in DNA precipitation with ethanol solution. This procedure is based on a molecular biology concept, where DNA purification is carried out in cold ethanol solution, by salt dissolving and DNA precipitation.

The following protocol adapted from Protocol-online (2012) was performed for DNA precipitation with ethanol:

1. Measure the volume of the oligonucleotide solution to be precipitated;
   Perform the following calculations:
   i. **Volume of 3 Molar Sodium Acetate pH 5.2** (see *Appendix III*) = 0.1*Volume of oligonucleotide solution
   ii. **Volume of 95% Ethanol** = 2.5* (Volume of oligonucleotide solution + Volume of 3 Molar Sodium Acetate pH5.2)
2. Add the calculated volume of 3 M Sodium Acetate into the oligonucleotide solution and vortex for 10 seconds to mix;
3. Add the calculated volume of 95% Ethanol into the oligonucleotide solution and vortex for 10 seconds to mix (prior to use, the ethanol should be chilled in freezer at -20ºC or, in alternative, on dry ice);
4. Place the reaction mixture in the freezer at -20ºC (or, in alternative, on dry ice) for at least 1h;
5. Centrifuge (20000 rpm, 20 min at 4ºC) the reaction mixture, in order to obtained a pellet of the precipitated oligonucleotide;
6. Pipette off the supernatant and discard;
7. Add the same volume of cold 95% Ethanol to the pellet and vortex;
8. Decant or carefully pipette off the supernatant

9. Repeat Step 7 and 8, once more;
10. Dry the pellet (tubes open, ~15 minutes at 37 ºC) on an incubator and, 15 minutes more on a desiccator;
11. Resuspend on 1x TE solution (10mM Tris + 1mM EDTA, pH 7.5-8) in final volume of 600 µl.

2.3. RESUSPENSION AND STORAGE OF OLIGONUCLEOTIDES

After the sulfhydryl activation and purification of the DNA Probe, the final concentration of the oligonucleotides was calculated by spectrophotometry based on the absorbance at 260 nm obtained with a *Perkin Elmer® UV-Vis* spectrophotometer (see *Appendix IV*).

The final concentrations of the DNA thiol Probe was calculated based on the absorbance measured at A_{260} using the Equation [4] (see *Appendix IV*):

$$Stock\ Conc.\left(\frac{\mu g}{\mu l}\right) = \frac{A_{260}}{l \times \varepsilon_{260}} \times Dilution\ factor \qquad [4]$$

where,

A_{260}= Absorbance peak at 260 nm
l= 1 cm of quartz cuvette
ε_{260}= Extinction coefficient, ~33 for *ss*DNA

Subsequently, both DNA thiol Probe and Target sequences, were diluted with 1x TE buffer (10mM Tris-CL +1 mM EDTA, pH 7.5-8.0) and aliquoted at a final desired concentration of 0.25, 0.50, 1.00 and 2.00 µM in a final volume of 250 µl (see *Appendix V*), wrapped in aluminium foil (to avoid light degradation of the fluorochromes) and stored at –20°C.

3. PREPARATION OF THE GOLD (AU) ELECTRODES

Before starting any assay in QCM-D (*Q-Sense® E1*) the Au electrode surface (*Q-Sense® QSX 301*, (*5MHz, 14 mm diameter, polished, AT-cut*)) has to be cleaned.

This procedure avoids the presence of organic material and contaminants on the electrodes surfaces and thus, ensures the maximum rate of immobilized and hybridized sequences.

The following protocol was used for Au electrodes ultra-cleaning:

1. Place the electrodes with the top upward, on a *BioForce ProCleaner™* Ultra-Violet and Ozone (UV/Ozone) chamber for 30 minutes;
2. The electrodes should afterwards be placed in a 5:1:1 solution of Milli-Q water/Ammonia/ Hydrogen Peroxide 25%, respectively, for 5 minutes in a bath at 75°C;
3. Dry the electrodes with a N_2 spray gun;
4. Place the electrodes into the UV/Ozone chamber for another 30 minutes;
5. Store in their respective box.

4. *E. COLI* O157:H7 MASS-SENSITIVE ANALYSIS WITH PIEZOELECTRIC TRANSDUCER ON AU ELECTRODE BY QCM-D DEVICE

In order to optimize the biological protocol to detect *E. coli* O157:H7 on Au electrodes by QCM-D device (*Q-Sense E1*), the following studies were made separately and sequentially, varying different parameters, such as, concentration and incubation time, on the steps of:

- DNA thiol probe immobilization;
- Blocking agent utilization, MCH, during SAM formation;
- DNA target hybridization.

In the final step of hybridization, the influence of the temperature was also studied.

Additionally, co-immobilization assays of the DNA thiol Probe and MCH blocking agent were also made, in order to verify in the present study, if there is an increase of hybridization efficiency.

4.1. *E. COLI* O157:H7 DNA THIOL PROBE IMMOBILIZATION WITH SAM FORMATION

A total of eight different immobilization assays, designated as *I*, using DNA Probe solutions with different concentrations (0.25, 0.50, 1.00 and 2.00 μM), and two immobilization times (30 and 60 minutes) were performed.

Table 5 shows the assays that were carried out, aiming to optimize the concentrations and immobilization time of the DNA thiol Probes at 22ºC.

Table 5: Representation of the assays carried out, to optimize the concentration and incubation time of the DNA thiol Probes immobilization at 22ºC.

		Time (min)	
		30	**60**
Concentration (µM) DNA thiol Probes	**0.25**	I_1	I_2
	0.5	I_3	I_4
	1.0	I_5	I_6
	2.0	I_7	I_8

4.1.1. DNA PROBE MASS IMMOBILIZATION PROTOCOL ON QCM-D

The DNA Probe mass immobilization assays on the QCM-D device were always performed at 22ºC, with the following protocol using the *Q-Sense* software:

1. Milli-Q water (18.2 MΩcm; Milipore, Tokyo) baseline performed with 5 minutes of rinsing at v= 25 (0.364 ml/min) followed by 5 min at v= 0, in order to obtain a stable zero baseline;
2. 1x TE buffer baseline performed with 5 minutes of rinsing at v= 25 (0.364 ml/min) followed by 5 min at v= 0, in order to obtain a stable reference baseline for the DNA mass calculation;
3. DNA thiol Probe mass immobilization was made with a final volume of 250 µL of a test concentration (0.25, 0.50, 1.00 or 2.00 µM) at v= 45 (0.644ml/min). After the introduction of the total volume of the DNA thiol Probe, the QCM-D device was stopped during the incubation time (30 or 60 minutes) and the immobilization phenomenon was recorded;
4. 1x TE buffer rinse step was performed with 10 minutes at v= 25 (0.364ml/min), in order to release the non-specific adsorbed DNA thiol Probes on the Au electrode surface.

After recording the variation of the frequencies (Hz) in the immobilization assays, using the *Q-Sense* software from QCM-D device, the data results were exported to *Qtools* software and converted in mass (ng/cm^2) by the Sauerbrey equation. To calculate

the immobilized mass of each assay, it was used the recorded frequency in the beginning point of the DNA thiol Probe immobilization and the last frequency point recorded of the rinsing step with 1x TE buffer.

4.2. MCH - BLOCKING AGENT DURING SAM FORMATION

After establishing the best conditions regarding, concentration and incubation time in the step of DNA thiol Probe immobilization, the next stage was the determination of the best conditions (concentration and incubation time) in the step of MCH blocking agent addition.

Typically, the preparation of MCH blocking agent solution is made by adding Milli-Q water, in order to obtain the desired concentration. However, in the present study, due to the required rising steps to calculate the mass blocked by MCH during SAM formation, it was predicted that the use of buffer 1x TE, would be more indicated. The reason for this assumption, is because the Milli-Q water rising steps would interfere with the precise calculation of the immobilized DNA thiol Probe mass, which were made in TE buffer solution, and bringing an added problem in the co-immobilization assays, with DNA thiol Probes and MCH blocking agent.

To verify if the use of 1x TE buffer would be possible or not, four assays were made (designated as $B_{W,}$ for the Milli-Q water and $B_{TE,}$ for the TE buffer), using MCH diluted in a concentration of 1.00 mM and two incubation times (30 and 60 minutes) carried out at 22ºC, as shown in Table 6.

Table 6: Representation of the assays performed to verify the use of 1x TE buffer instead of Milli-Q water, at 22ºC.

		Time (min)	
		30	**60**
Concentration 1 mM	**Milli-Q water**	B_{W1}	B_{W2}
	Buffer 1x TE	B_{TE1}	B_{TE2}

After the analysis of the MCH blocking assays with TE versus Milli-Q water, performed as Table 6, a total of more four assays (designed as *B*), were performed in order to determinate the best concentration (0.50 and 1.00 mM) and incubation time (30 and 60 minutes) at 22ºC, as shown in Table 7.

Table 7: Assays (designed with letter *B*) carried out to optimizing the concentration and incubation time of the MCH blocking agent at 22ºC.

		Time (min)	
		30	**60**
Concentration (mM)	**0.5**	B_1	B_2
	1.0	B_3	B_4

4.2.1. MCH MASS BLOCKING PROTOCOL ON QCM-D

MCH mass blocking assays on QCM-D device were always performed at 22ºC, with the following protocol using the *Q-Sense* software:

1. Baseline with the same solution as the one used to prepare MCH (Milli-Q water or 1x TE buffer), during 5 minutes at v=25 (0.364ml/min) followed by 5 minutes at v=0, in order to record a stable baseline;
2. MCH solution was introduced on a final volume of 250 µl at a desired concentration (0.5 or 1 mM), and v=45 (0.644 ml/min). The incubation time (30 or 60 minutes) was performed at v=0, in order to register the MCH mass blocking around of the DNA thiol Probe;
3. Rinsing step of 10 minutes with the prepared base solution of MCH at v=25 (0.364ml/min), in order to release the non-specific MCH blocked mass and non-immobilized DNA thiol Probes.

After recording the variation of the frequencies (Hz) in the MCH blocking agent assays, using the *Q-Sense* software from QCM-D device, the data results were exported to *Qtools* software and converted in mass (ng/cm^2) by the Sauerbrey equation. To calculate the MCH blocked mass of each assay, it was used the recorded frequency in

the beginning point of the MCH blocking agent detection and the last frequency point recorded of the rinsing step with 1x TE buffer.

4.3. *E. COLI* O157:H7 TARGET DNA HYBRIDIZATION

The hybridization of the target DNA represents the final step to be considered in the optimization of the biological protocol to detect *E. coli* O157:H7 by a QCM-D device.

In this step the key parameters to obtain a good hybridization signal will be not only the concentration of the complementary DNA, but also the hybridization temperature.

Table 8 shows the five different hybridization assays (designated as *H*) that were carried out, to establish the best conditions of target DNA concentration (0.50 and 1.00 μM) and hybridization temperature (22 and 30ºC).

Assays H_1 and H_2 were performed to evaluate the concentration of the target DNA that gave best results.

Based on the results of these two assays, the parameter "incubation temperature" was evaluated by assay H_4.

Besides these assays, considering the information available in the literature, co-immobilization tests were performed, in the attempt of achieving a signal as intense as possible to be detected by QCM-D device, during the hybridization step.

Two co-immobilization assays were performed in order to evaluate the influence of the temperature parameter, namely assays H_3 and H_5, conducted at 22ºC and 30ºC respectively.

Table 8: Representation of the assays carried out to obtain the best concentration and temperature parameters in order to optimize the hybridization signal and detection by QCM-D.

	Time (min)	**T (ºC)**
Type of Assay	**60**	
DNA Probe + MCH+ 1.0 μM Target	H_1	22
DNA Probe + MCH+ 0.5 μM Target	H_2	22
[DNA Probe + MCH] + Best concentration Target	H_3	22
DNA Probe + MCH + Best concentration Target	H_4	30
[DNA Probe + MCH] + Best concentration Target	H_5	30

4.3.1. DNA MASS HYBRIDIZATION PROTOCOL ON QCM-D

The DNA hybridization assays on QCM-D device were performed at 22ºC in the H_1, H_2 and H_3 assays or at 30ºC in the H_4 and H_5 assays, with the following protocol, using the *Q-Sense* software:

1. 1x TE buffer baseline of 5 minutes at v=25 (0.364ml/min) followed by 5 minutes at v=0; this step was made in order to obtain a stable baseline;
2. DNA Target (0.50 or 1.00 μM) hybridization was performed with a final volume of 250 μL at v= 45 (0.644ml/min) and desired temperature (22 or 30ºC). After the total introduction of the DNA Target, the QCM-D device was stopped during the incubation time (60 minutes) and the hybridization phenomenon was recorded;
3. 1x TE buffer rinse step was performed during 10 minutes at v= 25 (0.364ml/min), in order to release the non-specific hybridized DNA target.

After recording the variation of the frequencies (Hz) in the hybridization assays, using the *Q-Sense* software from QCM-D device, the data results were exported to *Qtools* software and converted in mass (ng/cm^2) by the Sauerbrey equation. In order to calculate the hybridized mass of each assay, it was used the recorded frequency in the beginning point of the DNA target hybridization and the last frequency point recorded of the rinsing step with 1x TE buffer.

5. EPIFLUORESCENCE MICROSCOPY ANALYSIS FOR QCM-D DATA VALIDATION

In order to validate the QCM-D data results, an inverted microscope system (NIKON®, Elipse T*i*) with epifluorescence and coupled with a HAMAMATSU® digital camera was used, with the exception of the electrodes used in the hybridization assays, which were observed using the Epifluorescence microscope coupled to a NIKON® colour camera.

Since the Epifluorescence microscope was shared equipment in the CEIT centre, the observation of the fluorescence labels in the Au electrode were typically made, between one at three days after the QCM-D assays. In the meantime, the Au electrodes were stored at 4ºC covered in aluminium foil, in order to prevent the fluorochrome degradation by direct light.

MCH blocking assays, at different concentrations and time incubation, were not visualized on the Epifluorescence microscope, because it was considered that they would not have relevance in the direct visualization of the pre-immobilized amount of DNA probes.

5.1. FLUOROCHROMES, FILTERS AND SPECTRUM USED

For the microscope observation of the emitted fluorescence by the DNA thiol Probe and Target, three types of filters were used, namely:

- ***FITC:*** enabled the excitation and emission of the fluorochrome FITC present on the DNA thiol Probe;
- ***TxRd:*** enabled the excitation and emission of the fluorochrome TxRd present on the DNA Target;
- ***Cy3-Cy5:*** enabled the excitation and emission of both fluorochromes, FITC and TxRd, when DNA thiol Probes and Target are hybridized.

Figure 11 shows the fluorochromes, *Fluorescein isothiocyanate* (FITC), emitting green light, and *Texas red* (TxRd), emitting red light, typically used in modified DNA strands, and used in the present work, in order to verify the data mass results of immobilization and hybridization recorded in QCM-D device.

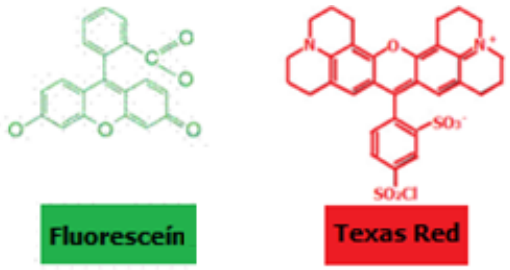

Figure 11: Scheme of the fluorochromes Fluorescein and Texas Red. [Adapted from: NCBI (c), 2012]

Table 9 shows the maximal absorbance and emission wavelengths for the fluorochromes used in the present work.

Table 9: FTIC and TxRd max absorbance and Emission wavelengths. [Adapted from: Sigma-Aldrich, 2012]

Fluorochrome	MW (Daltons)	Max Absorbance (λ, nm)	Max Emission (λ, nm)
Fluorescein	389	492	520
Texas Red	625	595	615

Table 10 shows the average transmission, centre of the wavelength and bandwidth for the single-band exciter and emitter of the three filters used on the Epifluorescence microscopy analysis.

Table 10: Characteristics of the exciter and emitter filters, used in the present work in the epifluorescence microscope.

Filter	Average transmission	Centre wavelength (λ, nm)	Bandwidth (nm)
FITC single-band exciter	> 93%	482	35
FITC single-band emitter	> 93%	536	40
TxRd single-band exciter	> 93%	562	40
TxRd single-band emitter	> 93%	624	40
Cy3-Cy5 dual-band exciter	> 90%, 90%	534, 635	36, 31
Cy3-Cy5 dual-band emitter	> 90%, 90%	577, 690	24, 50

Comparing Table 9, namely the maximal absorbance and emission wavelengths for the used DNA fluorochromes and Table 10, average transmission for single-band exciter and emitter for the used filters, it can be observed that the FITC and TxRd filters had a good average transmission (> 93%) when used separately to detect the mass immobilized or hybridized in the Au electrodes, respectively. However, for the Cy3-

Cy5 filter, which represents the dual-band exciter and emitter bandwidth for the FITC and TxRd fluorochomes when conjugated together (Table 10), does not coincide with the maximal absorbance and emission wavelengths of the fluorochromes (Table 9), which means that the average transmission will be less than 90%. Because of this fact, the detected fluorescent signal after hybridization (DNA thiol Probes and Targets) is expected to be lower than the separated fluorescence signals performed with the above single-band filters.

6. DETERMINATION OF THE FLUORESCENCE MASS BY *IMAGEJ* SOFTWARE

In order to verify the QCM-D mass immobilized and/or hybridized results, to the fluorescence images taken in Epifluorescence microscope, it was used some image processing functions of the *ImageJ 1.45s* [64 bits] software.

This software enabled the segmentation of the recorded fluorescence images, allowing the manual choice of the fluorescence area pixels and discarding the pixels from the images background. Some of these background pixels are derived from noise figure stablished on the semiconductors of the epifluorescence microscope image sensor. The segmentation was performed selecting, manually, the histogram dynamic range that was associated to the fluorescence regions. As these images are colour images they were processed concerning three histograms: the hue histogram, the saturation histogram and the brightness histogram.

After the segmentation process, the detected regions (fluorescence regions) were filtered. This stage is important to eliminate, for instance, some small regions (formed by few pixels that could represent noise). Other features were used to filter unwanted regions (e.g. the region circularity). At the final stage, the software creates a statistical map about the selected regions. It counts the total number of the selected fluorescence areas (*Count*), as well as, total area in pixels, of the selected regions (*Total Area*), average size of the regions (*Average Size*) and total area fraction that compares the image area to the selected areas (*Area Fraction*). In this way, knowing the total area fraction of the selected regions (fluorescence regions) represent in the total area, it is possible infers, roughly, about the immobilized mass.

An example of the procedure made with *ImageJ* software for the images segmentation, is demonstrated in *Appendix VI*.

CHAPTER IV- RESULTS AND DISCUSSION

1. DETERMINATION OF DNA THIOL PROBE CONCENTRATION BY SPECTROPHOTOMETER ANALYSIS

After the sulfhydryl activation and purification of DNA thiol Probe, the spectrophotometer *Perkin Elmer® UV-Vis* was used in order to verify the final concentration of the oligonucleotides.

Figure 12 shows the UV-Vis spectra obtained between 200-700 nm, for DNA thiol Probe after the sulfhydryl activation and purification.

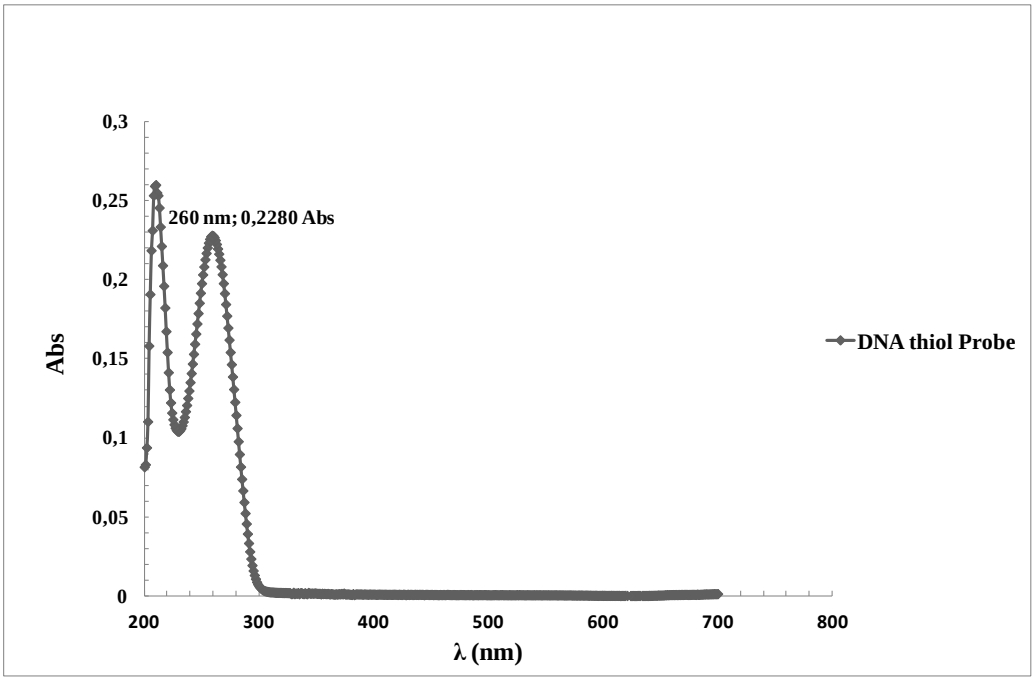

Figure 12: UV-Vis spectra obtained for the DNA thiol solution.

Figure 12 reveals that the DNA thiol solution spectra had two peaks between 200-700 nm, one at 210 nm, and the other at 260 nm.

It is remembered that, nitrogenous bases in nucleotides have an absorption maximum at about 260 nm and the carbonyl group of the amide bond absorbs at 190-210 nm (Nguyen and Ward, 1993). For this reason, the DNA concentration is generally determined by UV absorbance at 260 nm (Somma, 2006). Based on this premise, the DNA absorbance was recorded at 260 nm and corresponded to 0.2280 Abs.

Using Equation [4] (see *Appendix IV*), the DNA thiol Probe concentration was calculated, and a value of 0.697 μg/μl was obtained, corresponded to 96.6 μM in a final volume of 600 μl. This result indicates that a 61.10% of DNA Probe was recovered,

which is a lower percentage than the proposed methods by NAP-10 or Sephadex column (>90%) (GE Healthcare, 2006).

Another interesting result that should be referred is the absence of a peak at 283 nm which corresponds to the absorbance wavelength of the reagent DTT in the oxidized form (Iyer & Klee, 1973). This result indicates that the DNA thiol Probe was successfully reduced with the DTT solution and that the used DNA precipitation by ethanol protocol, allowed the successful removing of the DTT reagent in excess and other reaction byprodutcs.

Although the values obtained for recovery % of the DNA molar mass were not very high, the selected procedure of DNA precipitation by ethanol allowed obtaining DNA with high purity and revealed to be a less expensive method compared to chromatographic approaches.

2. *E. COLI* O157:H7 MASS-SENSITIVE ANALYSIS WITH PIEZOELECTRIC TRANSDUCER ON AU ELECTRODE BY QCM-D DEVICE

After recording the frequency and dissipation variation, in real time, of each one of the different assays using the *Q-Sense* software of the QCM-D device, the obtained data were exported to the software *Qtools*, to be further analyzed.

As previously referred in *Chapter II- Bibliography Review*, with the *Qtools* software the Sauerbray equation (Equation 1) can automatically be used, relating the Frequency variation (Hz) of the recorded assays in mass (ng/cm^2), where the decrease of frequency represents the mass deposition and the increase represents the release of the mass deposited.

Based on this relationship and using the *Qtools* software, the following results were obtained.

In all the assays performed in this study the variation of the Dissipation energy was always less than 1×10^{-6} per 10 Hz, which indicates the existence of a rigid film immobilized and/or hybridized in the Au electrode. Due to this fact, the Dissipation energy is not represented in the following Tables, were mass values results are presented.

2.1. *E. COLI* O157:H7 DNA THIOL PROBE IMMOBILIZATION WITH SAM FORMATION

As referred in the *Chapter III- Material and Methods*, a total of 8 immobilization assays, testing different parameters were performed. As an example, Figure 13 represents the obtained results of an immobilization assay performed in QCM-D. The figure shows the real-time recording of the variation in Frequency (Hz) represented in blue colour and the Dissipation ($1x10^{-6}$) represented in orange colour, recorded with the 9^{th} overtone with the QCM-D E1 device.

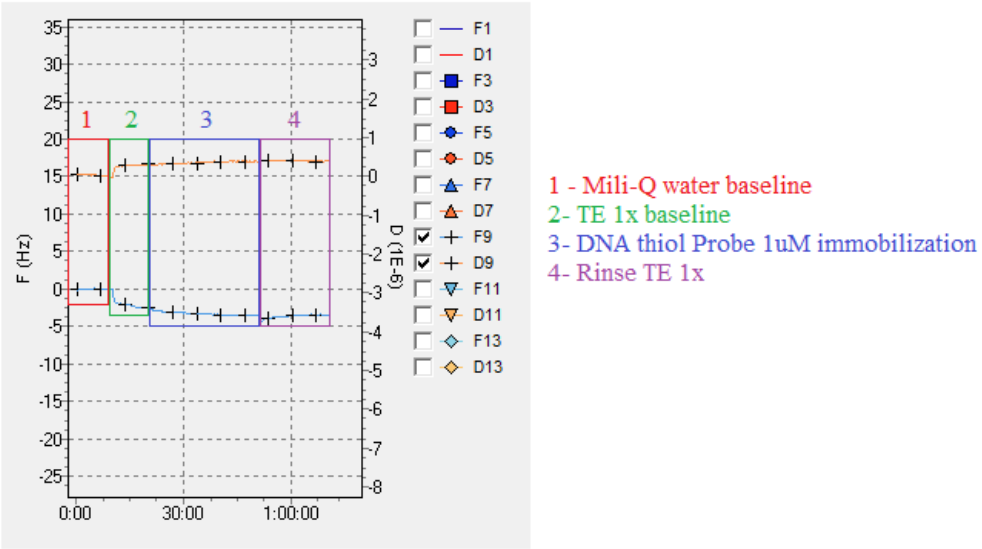

Figure 13: Graphical representation of an I_5 immobilization assay performed at 22ºC, in *QCM-D E1* device and recorded with the 9th overtone.

The first step, showed in the red block colour, represents the recorded Frequency of the zero baseline, using Milli-Q water.

The second step, showed in the green block colour, represents the variation of the Frequency, which is lower than the previously obtained during the Milli-Q water step, due to the presence of salts in the TE buffer.

The third step, showed in the blue colour represents the DNA thiol Probe immobilization (in the example using a concentration of 1.00 µM and during 30 minutes of incubation time). In this third step it can be seen that the Frequency is lower than the TE baseline, showing the immobilization of the DNA thiol Probe mass in the Au electrode.

The fourth step, represented by violet colour, shows the rinsing step with the TE buffer, which removed the DNA probes that were not specific immobilized.

Table 11 shows the results of the observed mass immobilization on the different assays, carried out in the present work (results are showed in the same order as the assays presented on Table 5, in *Chapter III- Materials & Methods*,). The results are presented in triplicate for each assay, together with the Average ($\overline{x}$) and Standard Deviation (σ) of the values. As referred in the material and methods section, for each assay at least 3 replicates were performed, nevertheless for some assays more than 3 replicates were needed to obtain coherent results. The replicate is indicated in table 11

as the second number on the index (e.g. $I_{3.6}$. means 3rd Immobilization assay, 6th replicate).

Table 11: Representation of triplicate results of the different mass immobilization assays (ng/cm^2), carried out on the present work.

		Incubation Time(min)			
		30		**60**	
		Assays	**Mass (ng/cm^2)**	**Assays**	**Mass (ng/cm^2)**
Concentration (µM)	**0.25**	$I_{1.1}$	8.04	$I_{2.3}$	11.77
		$I_{1.3}$	0.74	$I_{2.4}$	9.93
		$I_{1.4}$	6.38	$I_{2.9}$	12.80
		$\bar{x}$	**5.05**	$\bar{x}$	**11.50**
		σ	**2.88**	σ	**1.05**
	0.5	$I_{3.1}$	8.68	$I_{4.1}$	20.58
		$I_{3.4}$	29.53	$I_{4.5}$	19.49
		$I_{3.6}$	9.72	$I_{4.6}$	10.60
		$\bar{x}$	**15.98**	$\bar{x}$	**16.89**
		σ	**9.04**	σ	**4.19**
	1.0	$I_{5.1}$	25.14	$I_{6.3}$	6.26
		$I_{5.3}$	18.83	$I_{6.5}$	2.60
		$I_{5.4}$	10.20	$I_{6.6}$	29.34
		$\bar{x}$	**18.06**	$\bar{x}$	**12.73**
		σ	**5.24**	σ	**11.07**
	2.0	$I_{7.3}$	6.47	$I_{8.2}$	8.90
		$I_{7.5}$	20.43	$I_{8.4}$	29.61
		$I_{7.6}$	4.25	$I_{8.5}$	14.19
		$\bar{x}$	**10.38**	$\bar{x}$	**17.57**
		σ	**6.70**	σ	**8.03**

By observing the results presented on Table 11 it can be observed that, to obtain three valid results for each assay in the QCM-D, sometimes a several number of replicates had to be made. This can be ascribed to different reasons, such as, the unstable baselines recorded in some cases, in other cases, an increase of frequency was recorded during the step of DNA thiol Probe which would be translated into a negative

mass immobilization, and/or decrease of frequency in the rinsing step with 1x TE buffer.

The results obtained for all the replicates performed for each assay, are presented in *Appendix VII*.

Table 11 also shows that the assay with the maximum average value for mass immobilization is the one represented by I_5 (1.00 μM of DNA thiol Probe at 30 minutes of incubation time).

The Standard Deviation shows the high deviation between the mass immobilization recorded on the repeated tests in almost all assays. This difference in mass immobilization can be explained by numerous factors, including, variations of environmental conditions between assays (temperature, humidity), and physical conditions of the Au electrodes, among others.

In order to facilitate the evaluation of the obtained values, the results presented on Table 11 are also exposed in a graphic format (Figure 14).

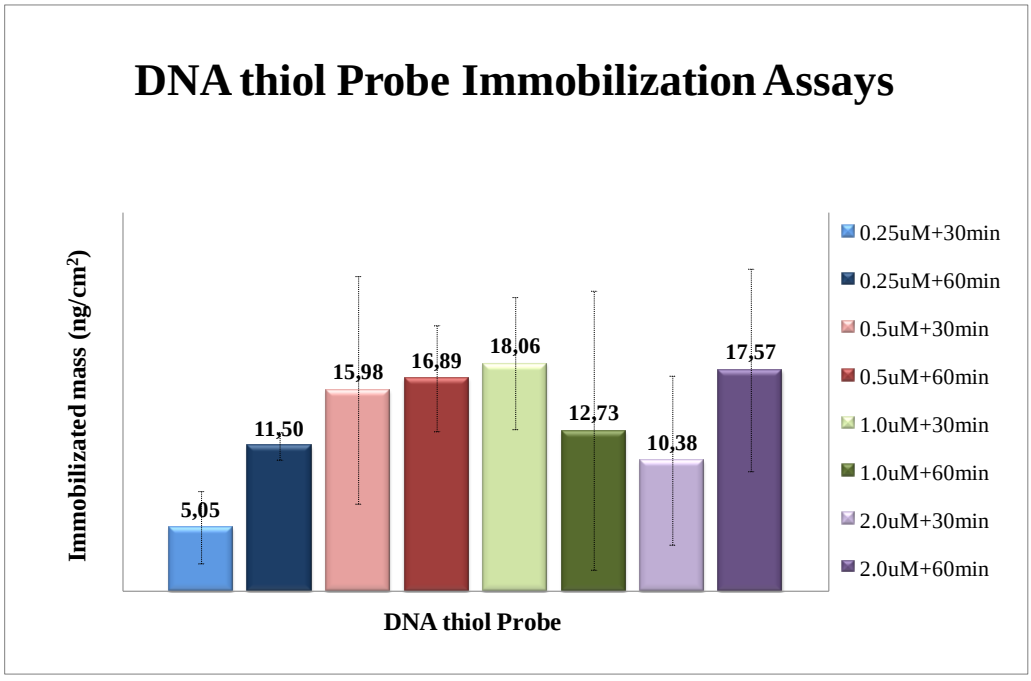

Figure 14: Graphical representation of the average and Standard deviation obtained for the DNA thiol probes immobilization assays at different concentrations and incubation time.

Figure 14 shows once more that, the higher average values obtained for the DNA thiol Probe mass immobilization were attained in assays I_3, I_4, I_5 and I_8, with assay I_5 (performed with a concentration of 1.00 μM at 30 minutes), giving the best results, with a total average of 18.06 ng/cm^2. The graphic also allows observing that, although the four referred assays were the ones that gave higher average values for mass deposition,

for I_3 and I_8 the standard deviation values obtained were very high, pointing to a high variability in the obtained results, while lower deviations were achieved in assays I_4 and I_5. Therefore, conditions of assay I_5 were chosen to proceed to the next stages (evaluation of the blocking agent deposition and DNA target hybridization) of this work since it allowed obtaining the higher average value for DNA mass deposition on the Au electrode (18.06 ng/cm^2) with one of the lowest values for standard deviation.

2.1.1. Epifluorescence Microscopy analysis for DNA thiol Probe immobilization assay

After performing the DNA thiol Probe immobilization assays in the QCM-D device, the Au electrodes were observed by epifluorescence microscopy, as soon as possible.

Figure 15 shows a compilation of one image for each assay represented on Table 11, taken with the Epifluorescence microscope, as well as, the calculated average mass of DNA probe immobilized on the Au electrode.

Each one of these images represents the entire Au electrode, with a 10x objective and 10x ocular, on a 10x10 mosaic, using 2 seconds of light exposure and 13.60x of gain, with FITC filter using a HAMAMATSU® digital camera.

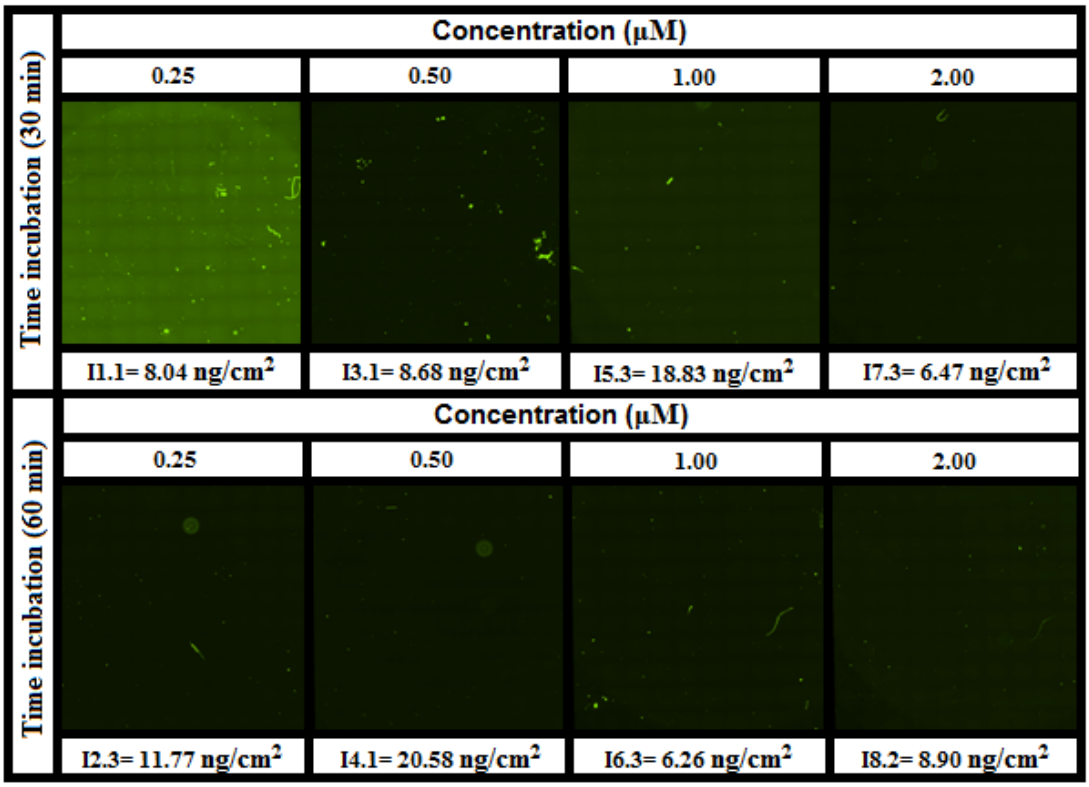

Figure 15: Comparison of immobilization results by QCM-D and Epifluorescence Microscope.

Observing Figure 15, there appears to be a disparity between the mass calculated from the Sauerbrey equation in the QCM-D device and the fluorescence visualized by Epifluorescence Microscopy.

In order to obtain a better appreciation of both methods results (QCM-D and Epifluorescence microscope images), the *ImageJ 1.45s* software was used as previously explained in *Appendix VI*.

The segmented images are shown in Figure 16.

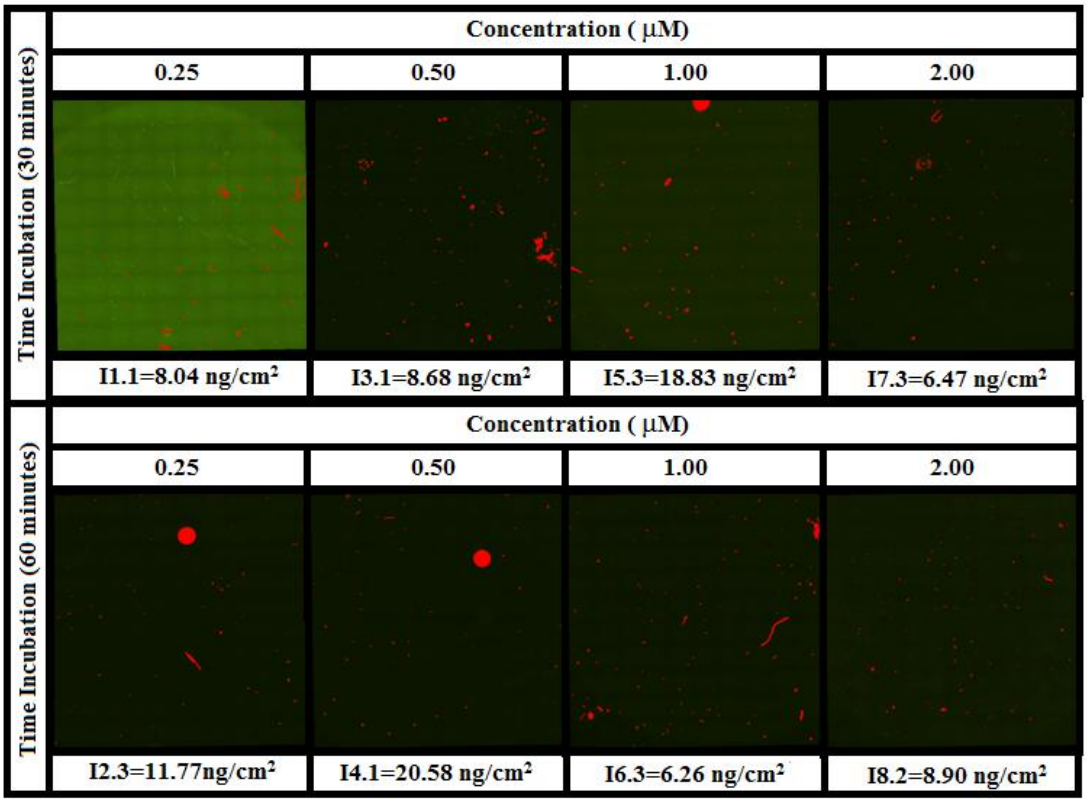

Figure 16: Comparison of results for the immobilization assays between QCM-D and Epifluorescence Microscope after the *ImageJ* software analysis.

Table 12 shows the average results for Count, Total Area (pixels), Size (pixels) and Area Fraction (% pixels) using the images of the immobilization assays taken by Epifluorescence microscope, with FITC filter and processed with *ImageJ* software, as well as, the Standard Deviation (σ) of the Area Fraction.

Table 12: Representation of the *ImageJ* processed images results for the immobilization assays.

Assay	Count	Total Area (pixels)	Average Size (pixels)	Area Fraction (%)	σ - Area Fraction
***I*$_1$ (0,25µM+30min)**	216,33	50095,67	275,77	0,27	0,15
***I*$_2$ (0,25µM+60min)**	764,67	60103,33	210,40	0,33	0,25
***I*$_3$ (0,5µM+30min)**	237,33	70199,00	265,55	0,37	0,31
***I*$_4$ (0,5µM+60min)**	437,67	88490,33	372,16	0,43	0,12
***I*$_5$ (1,0µM+30min)**	305,67	100828,67	366,02	0,53	0,21
***I*$_6$ (1,0µM+60min)**	294,00	59859,33	198,78	0,33	0,12
***I*$_7$ (2,0µM+30min)**	411,33	60383,33	153,88	0,30	0,00
***I*$_8$ (2,0µM+60min)**	548,33	85003,33	158,09	0,40	0,10

The Area Fraction (%) values, showed in Table 12, were calculated by relating the Total Area (pixels) with the total number of pixels of the each recorded image.

Table 12 shows that, the highest total area in pixels and consequently area fraction was obtained by the assays with I_5 (1.00 µM at 30 minutes of incubation time).

Figure 17 shows the graphical representation of the area fraction (%) for the assays results, previews showed in Table 12, as well as, the Standard Deviation of them.

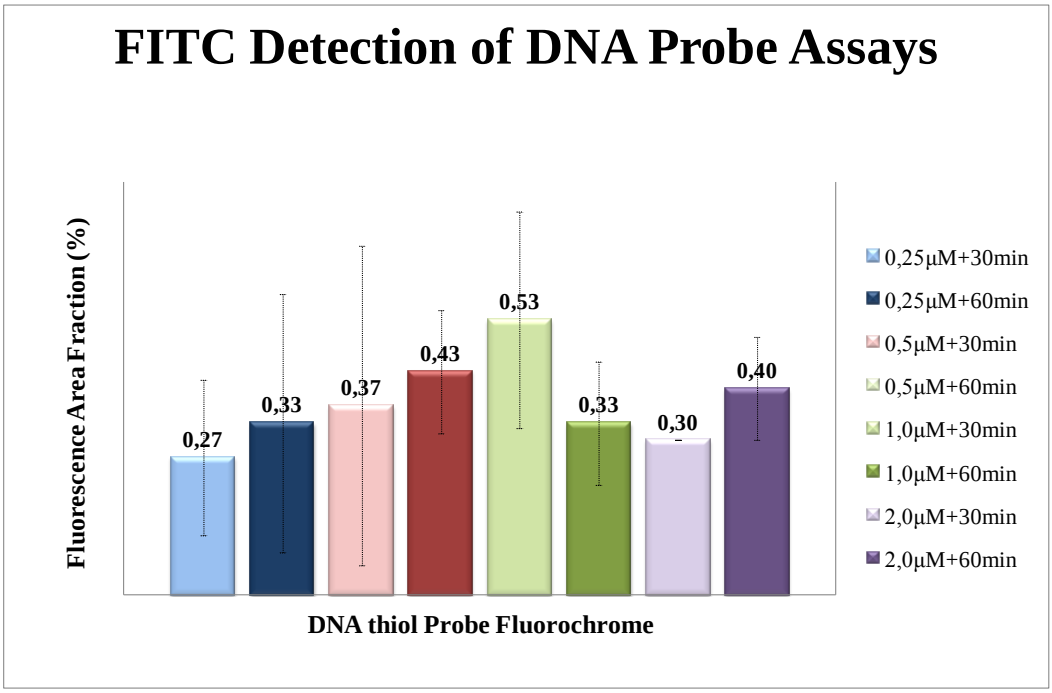

Figure 17: Graphical representation of the average and standard deviation of the fluorescence area fraction (%) in each assay.

The results presented in Figure 17 are in general in good agreement with those obtained with the QCM-D device. It can be observed, that the assay performed with

1.00 μM of DNA thiol Probe at 30 minutes of incubation time (I_5), shows the highest percentage of FITC fluorescence fraction area immobilized, when analyzing the images obtained by Epifluorescence microscopy and processed by the software *ImageJ* (total of 0.53%), which reinforces the choice of these parameters (1.00 μM DNA thiol Probe; 30 minutes incubation time) to be used in the following assays.

2.2. MCH - BLOCKING AGENT DURING SAM FORMATION

In order to verify the possibility of preparing the MCH blocking solution using 1x TE buffer instead of Milli-Q water, the assays showed in Table 6 (please see *Chapter III- Material and Methods*) were performed. Table 13 shows the obtained results of the DNA thiol Probe pre-immobilized mass and MCH blocking agent mass for each assay, conducted in triplicate, as well as, Average ($\overline{x}$) and Standard Deviation (σ) values.

Table 13: Results of pre-immobilized mass of DNA thiol Probe and MCH blocking agent mass (ng/cm^2) of the different assays.

MCH Blocking time (min)

MCH Concentration (1 mM)		30			60		
	Assay	DNA Mass (ng/cm^2)	MCH Mass (ng/cm^2)	Assay	DNA Mass (ng/cm^2)	MCH Mass (ng/cm^2)
Milli-Q water	$B_{W1.1}$	-44.62	79.32	$B_{W2.1}$	13.03	166.99
	$B_{W1.2}$	17.79	122.79	$B_{W2.2}$	-13.08	142.15
	$B_{W1.3}$	-1.50	167.69	$B_{W2.3}$	30.03	107.48
	Average	***17.79***	***122.79***	***Average***	***21.53***	***137.24***
	σ	-	-	σ	***12.02***	***42.08***
	Assay	DNA Mass (ng/cm^2)	MCH Mass (ng/cm^2)	Assay	DNA Mass (ng/cm^2)	MCH Mass (ng/cm^2)
Buffer 1x TE	$B_{TE1.1}$	14.58	134.12	$B_{TE2.1}$	11.65	51.06
	$B_{TE1.2}$	17.97	92.37	$B_{TE2.2}$	8.91	106.40
	$B_{TE1.3}$	18.75	108.45	$B_{TE2.3}$	5.94	103.39
	Average	***17.10***	***111.65***	***Average***	***8.83***	***86.95***
	σ	***2.22***	***21.06***	σ	***2.86***	***31.12***

Analysing the average value results, presented in Table 13, it can be observed that a concentration of 1.00 mM of MCH blocking agent prepared with Milli-Q water had, in both assays (30 and 60 minutes of blocking time) a higher blocked mass than the ones prepared with 1x TE buffer.

As presented before, grey results represent the negative values obtained on pre-immobilized DNA thiol Probe, which influenced the MCH blocked mass results, due to this reason they were discarded.

Figure 18 graphically represents the MCH mass values arising from the MCH blocking assays with Milli-Q water and 1x TE buffer, shown on Table 13.

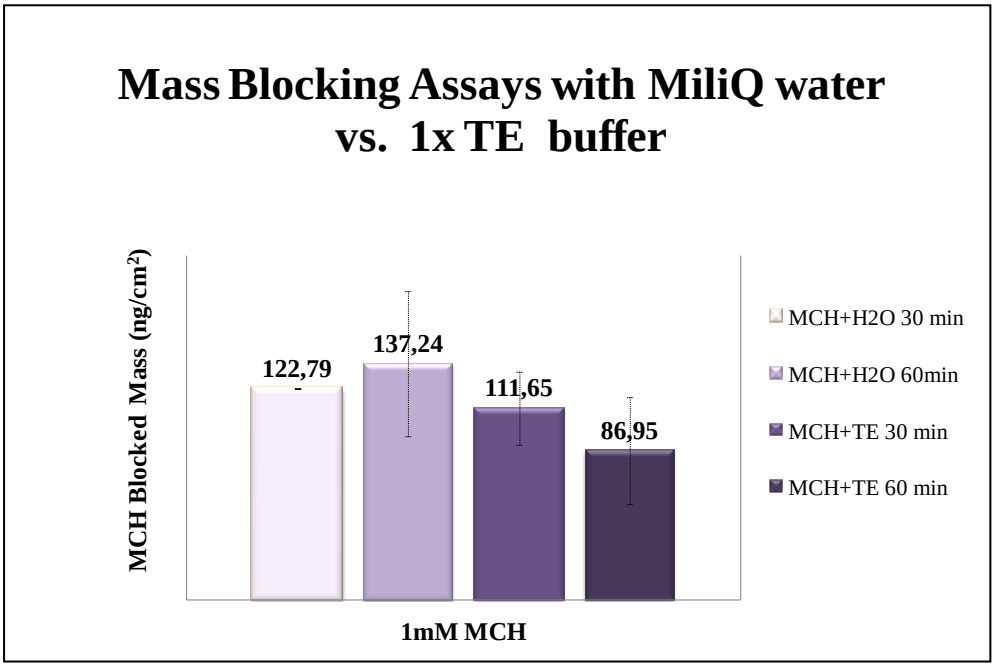

Figure 18: Graphical representation of the average values for the MCH blocking assays, with Milli-Q water and buffer 1xTE at different incubation time.

Figure 18 shows, that the highest MCH mass blocked was performed with the preparation of the blocking agent in Milli-Q water at 60 minutes of time incubation, with a total amount of 137.24 ng/cm^2.

However, the aim of these assays above, was to prove if the MCH blocking agent could be prepared with 1x TE buffer and if this blocking agent still works when prepared in this buffer.

The value results shown without doubts, that MCH blocking agent can be prepared in 1xTE buffer, even if the adsorbed MCH mass appears to be lower than with MCH prepared in Milli-Q water, for the studied conditions.

Based on these results the next performed study aimed to determine the best concentration and MCH blocking time when prepared with 1x TE buffer, as Table 7 showed in the *Chapter III- Materials and Methods*. The triplicate results for these assays, as well as the Average ($\overline{x}$) and Standard Deviation (σ) of the values, are shown in Table 14.

Table 14: Results of mass pre-immobilized DNA thiol Probe and MCH blocked (ng/cm^2) in 1x TE buffer assays, carried out at different concentration and incubation time.

		MCH Blocking time (min)					
		30			**60**		
MCH Concentration (mM)		**Assay**	**DNA Mass (ng/cm^2)**	**MCH Mass (ng/cm^2)**	**Assay**	**DNA Mass (ng/cm^2)**	**MCH Mass (ng/cm^2)**
0.5		***B*$_{1.1}$**	12.93	106.06	***B*$_{2.1}$**	18.74	98.33
		***B*$_{1.2}$**	9.71	123.72	***B*$_{2.2}$**	27.92	111.86
		***B*$_{1.3}$**	16.45	86.23	***B*$_{2.3}$**	8.74	94.87
		Average	***13.03***	***105.34***	***Average***	***18.47***	***101.69***
		σ	***3.37***	***18.76***	***σ***	***9.59***	***8.97***
		Assay	**DNA Mass (ng/cm^2)**	**MCH Mass (ng/cm^2)**	**Assay**	**DNA Mass (ng/cm^2)**	**MCH Mass (ng/cm^2)**
1.0		***B*$_{3.1}$**	14.58	134.12	***B*$_{4.1}$**	11.65	51.06
		***B*$_{3.2}$**	17.97	92.37	***B*$_{4.2}$**	8.91	106.40
		***B*$_{3.3}$**	18.75	108.45	***B*$_{4.3}$**	5.94	103.39
		Average	***17.10***	***111.65***	***Average***	***8.83***	***86.95***
		σ	***2.22***	***21.06***	***σ***	***2.86***	***31.12***

Table 14 shows that, when compared different concentrations of MCH (1.00 and 0.50 mM) prepared with 1x TE buffer and incubated at different blocking times (30 and 60 minutes), the assay performed as B_3 (1mM of MCH at 30 minutes of incubation time) had a higher MCH adsorbed mass, with a total average of 111.65 ng/cm^2.

The graphical representations of these average mass values of the MCH blocking assays, presented in Table 14, are shown in Figure 19.

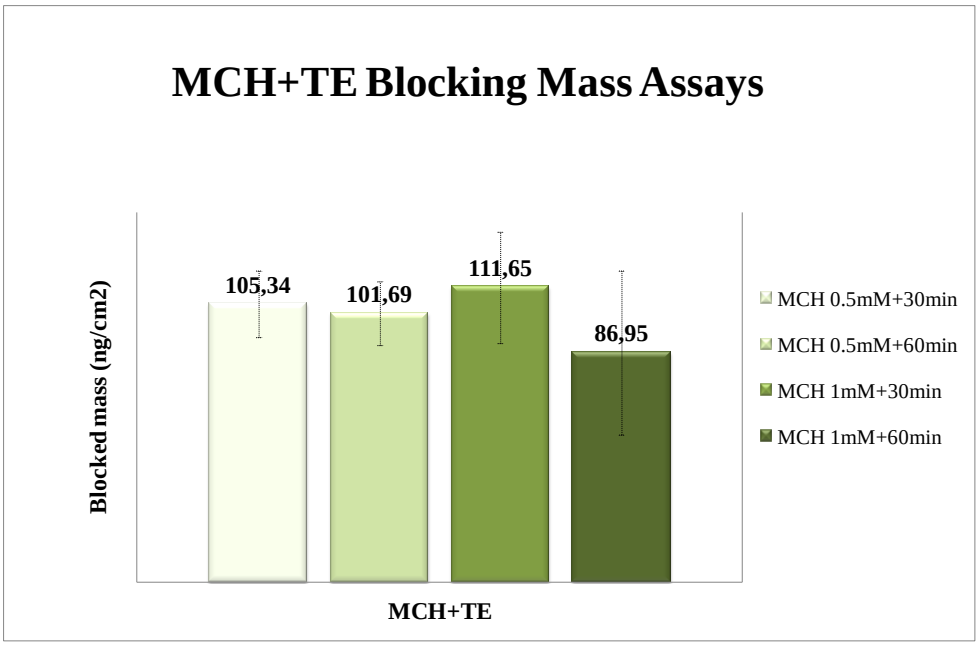

Figure 19: Graphical representation of the MCH mass blocking average when prepared with 1xTE buffer, at different concentration and time incubation.

As previously shown in Table 14, and graphically represented in the above Figure 19, the highest MCH blocking mass assay was performed as B_3 (1mM of MCH at 30 minutes of incubation time).

Based in these results, the step of MCH blocking agent was chosen to be performed using a final concentration of 1.00 mM of MCH (prepared with 1xTE buffer) during 30 minutes of incubation time, in the DNA thiol Probe SAM formation.

This choice also results advantageous for the final study of co-immobilization between the DNA thiol Probe and MCH blocking agent, because both required the same incubation time and buffer rinse.

2.3. *E. COLI* O157:H7 DNA TARGET HYBRIDIZATION

The final step, DNA Target hybridization study, was performed with the best assays results for the DNA thiol Probe immobilization (1.00 μM DNA thiol Probe at 30 minutes of incubation time) and MCH blocking agent (1.00 mM MCH prepared with 1x TE buffer solution at 30 minutes of incubation time), both performed at 22ºC.

2.3.1. DETERMINATION OF THE BEST DNA TARGET CONCENTRATION

Table 15 shows the first two hybridization values results (H_1 and H_2), based on Table 8 (please see *Chapter III-Materials and Methods*), and performed in triplicate at 22ºC, as well as, Average ($\overline{x}$) and Standard Deviation (σ) and Hybridization efficiency (%), intending to compare the best DNA Target concentration (0.50 or 1.00 μM) at 60 minutes of hybridization time.

Table 15: Representation of DNA thiol Probe mass immobilization, MCH mass adsorption and DNA target mass hybridization values results, obtained in QCM-D at 22ºC.

		Time incubation (min)		
		30		**60**
Concentration of DNA Target (μM)	**Assay**	**DNA Probe (ng/cm^2)**	**MCH agent (ng/cm^2)**	**DNA Target (ng/cm^2)**
1.0	***$H_{1.1}$***	10.27	147.47	- 4.65
	$H_{1.2}$	10.17	137.40	- 6.67
	$H_{1.3}$	4.54	13.68	0.85
	Average	***8.33***	***99.52***	***0.85***
	σ	***3.28***	***74.51***	-
	% Hybridization	***10.20***		
	Assay	**DNA Probe (ng/cm^2)**	**MCH agent (ng/cm^2)**	**DNA Target (ng/cm^2)**
0.5	***$H_{2.1}$***	35.01	140.45	- 8.71
	$H_{2.2}$	16.43	128.60	2.14
	$H_{3.3}$	-3.25	161.60	- 2.49
	Average	***25.72***	***134.53***	***2.14***
	σ	***13.14***	***8.38***	-
	% Hybridization	***8.32***		

Unexpectedly, Table 15 shows negative mass results, obtained in almost all H_1 and H_2 assays, when DNA Target was rinsing after the hybridization time. All these negative mass results happened when the rinsing steps were made. This negative results suggesting explanations, such as, the *ss*DNA Target were not specifically hybridized and the rising step had removed them; or the 1x TE buffer is not a good buffer for this work, which affect the mass determination (and also explain the negative results obtained for several times in the DNA thiol Probe immobilization); and another

explanation is the movement of the suction tubes in the exchange of solutions, that influenced the frequencies recording by the sensitive QCM-D device.

Comparing the Hybridization efficiency (%) in Table 15, the hybridization assay H_1 shows higher efficiency values than one performed as H_2 at 22ºC, respectively 10.20% to 8.32%.

However, even for this assay the hybridization efficiency shown a very low result.

Grey results represent, once more, the negative values obtained on mass values results, because of these fact they were discarded.

2.3.1.1. Epifluorescence Microscope analysis for DNA Target with concentration of 0.50 and 1.00 µM

After the DNA Target hybridization assays had been performed by QCM-D, the Au electrodes were stored at 4ºC, wrapped in aluminium, until they could be visualized on Epifluorescence microscope.

At this stage the HAMAMATSU® digital camera used before on Epifluorescence microscope, to visualize the DNA thiol Probes assays was damaged, and the alternative NIKON® colour camera was used.

In order to confirm the QCM-D mass hybridized results with the epifluorescence hybridized images, it was used, once again the *ImageJ 1.45s* software.

The stored images that were processed in *ImageJ* software to fluorescent area fraction determination (% pixels), represented the centre of the Au electrode, with a 10x objective and 10x ocular, on a 4x4 mosaic, using 2 seconds of light exposure and 13.60x of gain and manual focus.

This 4x4 mosaic images were used instead of the 10x10, because they were representative enough for the recorded fluorescence.

Table 16 reflects the comparison between the mass results (ng/cm^2) obtained with QCM-D device and the fluorescence area fraction (%) calculated for each assay.

Table 16: Representation of the average mass results obtained for the H_1 and H_2 assays, by QCM-D (ng/cm^2) and Epifluorescence Microscope (% area fraction).

	DNA Probe (ng/cm^2)	**FITC mass (% pixels)**	**DNA Target (ng/cm^2)**	**TxRd mass (% pixels)**	**Hybridization (%)**	**Cy3-Cy5 mass (% pixels)**
H₁	8.33	0.023	0.85	0.016	10.20	0.010
H₂	25.72	0.013	2.14	0.004	8.32	0.003

Unexpectedly, Table 16 does not show a direct relationship between the QCM-D mass results (ng/cm^2) and the correspondingly fluorescent area (% pixels) calculated by the software *ImageJ.* The explanation for these non-corresponding results can be made, due to the used NIKON colour camera, which was not specific to epifluorescence imaging and because of that, the taken images showed much lower fluorescence that when compared to the previews immobilization images, and also due to, the invalidation of most of the triplicated assays used in the mass calculation by the QCM-D device.

However, when the percentages of FITC and TxRd fluorescence total area are analyzed, H_1 shows a higher percentage than H_2. More important, the H_1 results shows once more, a higher percentage of specific hybridization calculated through the Cy3-Cy5 filter, as also is shown by the Hybridization efficiency (%) calculated through the QCM-D mass values.

Based on these results, the following hybridization assays were made with 1.00 μM of DNA target at 60 minutes of incubation time, in order to optimize others parameters for the detection of *E. coli* O157:H7.

2.3.2. Determination of the influence of temperature and co-immobilization into DNA Target mass hybridization

At this stage, three more hybridization assays were made in the QCM-D, in order to determinate if the co-immobilization of the DNA thiol Probe + MCH blocking agent and/or hybridization temperature at 30ºC, had a positive influence in the quantity of

target mass hybridized. These three last assays (H_3, H_4 and H_5) were performed under the conditions presented on Table 7 (please see *Chapter III-Materials and Methods*).

In order to allow a better understanding of the results achieved inthese assays, Figure 20 shows an example of each one (H_3, H_4 and H_5) performed with QCM-D device.

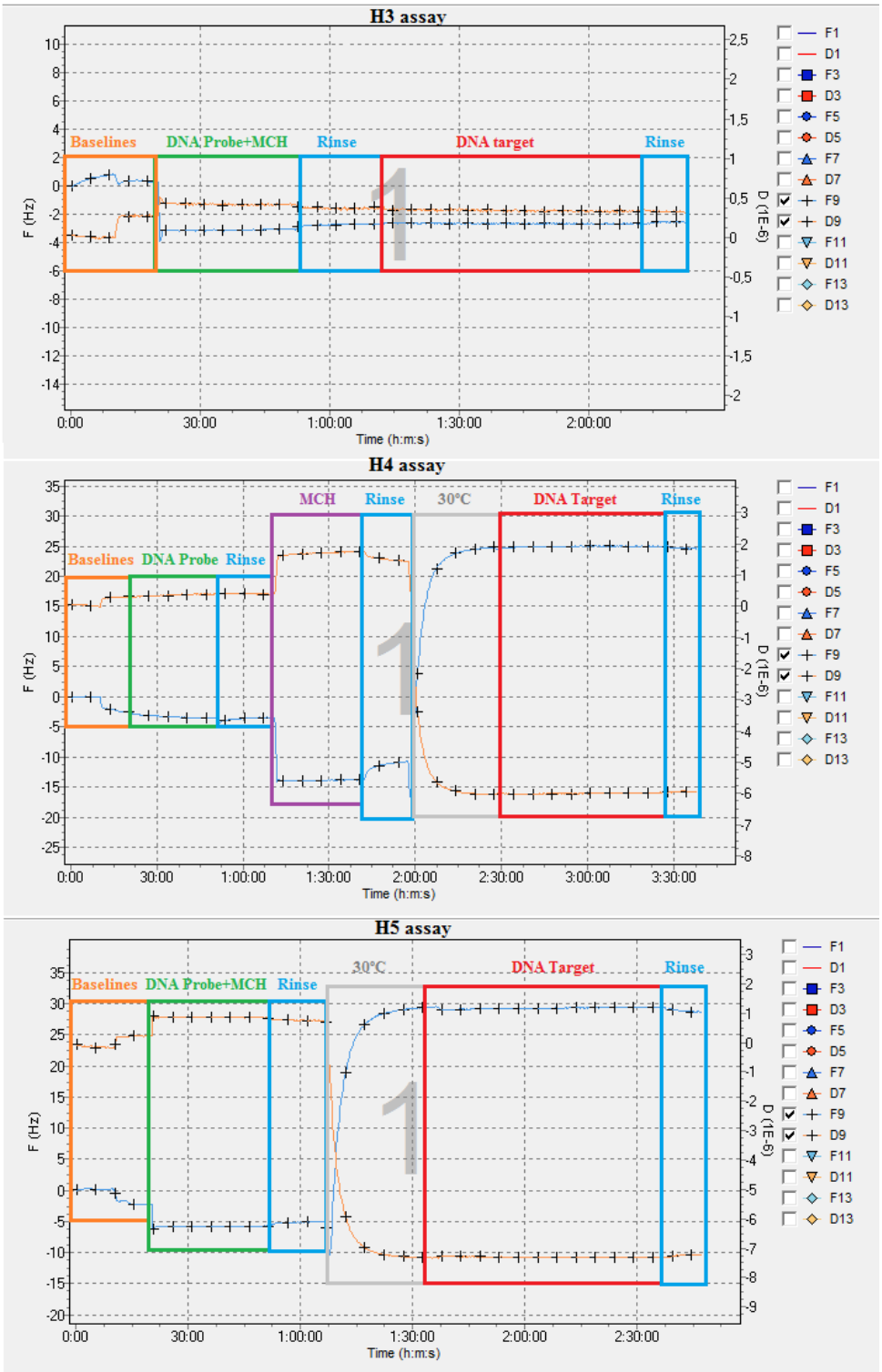

Figure 20: Examples of the results obtained for hybridization assays H_3, H_4 and H_5 performed with QCM-D.

Table 17: Representation of the comparison hybridization mass results on the H_3, H_4 and H_5 assays by QCM-D.

Hybridization Temp. (ºC)		**Time incubation (min)**		
		30		**60**
22	**Assay**	**DNA thiol Probe +MCH (ng/cm²)**		**DNA Target (ng/cm²)**
	$H_{3.1}$	48.83		- 6.84
	$H_{3.2}$	52.28		- 0.52
	$H_{3.3}$	138.61		- 3.93
	Average	***79.91***		-
	σ	***50.87***		-
	% Hybridization	-		
30	**Assay**	**DNA Probe (ng/cm²)**	**MCH agent (ng/cm²)**	**DNA Target (ng/cm²)**
	$H_{4.1}$	14.02	131.64	2.74
	$H_{4.2}$	12.46	120.40	4.72
	$H_{4.3}$	29.39	126.77	25.99
	Average	***18.62***	***126.27***	-
	σ	***9.36***	***5.64***	-
	% Hybridization	-		
30	**Assay**	**DNA thiol Probe +MCH (ng/cm²)**		**DNA Target (ng/cm²)**
	$H_{5.1}$	22.12		16.26
	$H_{5.2}$	46.82		14.54
	$H_{5.3}$	58.89		6.92
	Average	***42.61***		-
	σ	***18.74***		-
	% Hybridization	-		

Concentration of DNA Target (1 µM)

The assays were performed in triplicate and the obtained results were analyzed by the *Qtool* software. The calculation were made using the Sauerbrey equation, that uses the differences in Frequency (Hz), between the last recorded point in QCM-D (rinse step, represented in blue colour in Figure 20) with the initial recorded point in the hybridization step (represented in red colour in Figure 20).

In all the triplicate assays (H_1, H_2, H_3), the calculated hybridized mass was not considered to be a valid result.

As it can be observed (Table 17 and Figure 20), in the hybridization step of the H_3 assays, an increase of recorded frequency in the final rinsing step with the 1xTE buffer (blue colour) occurred, and thus a signal higher than the one of the hybridization frequency (red colour) was obtained (thus negative values were obtained and consequently discarded). These recorded results in QCM-D, suggests that the DNA target oligonucleotides were unspecifically hybridized and consequently released during the buffer rinse.

In the H_4 and H_5 assays, although positive values were obtained for the calculated mass deposition of DNA probes, it can be seen in Figure 20 that deposition occurred during the rinsing step, thus these values were also not considered as valid. In some assays, besides mass detection during rinsing steps, mass release during the incubation in the hybridization step was observed. These contradictory and unexpected results can possibly be due to the change of the hybridization temperature from 22ºC for 30ºC, as shown in Figure 20. Even though the QCM-D device was allowed to stabilize after temperature change, the results seem to point to a certain degree of instability and variability due to temperature changes in this sensitive equipment. It should be noticed that in the assays only the QCM device was programmed for 30 ºC, with the solutions maintained at ambient temperature. Thus, this small difference could possible interfere with the performance of the equipment. A possibility to avoid this interference would be the warming of the solutions (DNA target and rising buffer) to 30 ºC before being introduced in the QCM-D chamber. This was not attempted in the present work but it is a point that deserves further analysis in a future study.

Another possibility that can in part explain these unexpected results appears to be, once again, the choice of the buffer. As referred before, in this work it was chosen to use 1x TE as buffer, since it was recommended by *Sigma-Aldrich*. Nevertheless, this choice was probably not the best one, since in all immobilization and hybridization steps performed in the present study, minimal variation of recorded Frequency (always less than 2 Hz, which is almost the minimum limit detected by the QCM-D device) were observed. Cho and co-workers (2004) tested the effect of using two different buffers (1x TE and PBS) in a QCM device and concluded, that the "*surface density of immobilization probe molecules and the hybridization efficiency depending on the type of buffer and salt concentration*", and explaining that "*the amount of DNA probe*

adsorbed increased when the salt concentration is high because of the reduced electrostatic repulsion between anionic DNA strand." For this reason using a PBS buffer in QCM studies, appears to be a better buffer choice, resulting in a higher amount of DNA mass adsorbed, which in other hand gave to the operator more reproducible assays to compare. However the concentration of the PBS buffer is also a point to consider in future studies, since it was reported that it should be lower than 1M, because at this concentration PBS appears to conduce to an overestimated DNA mass adsorbed.

Due to these inconclusive hybridization results, obtained from the QCM-D software, the epifluorescence microscopy and its recorded images were used, in order to verify, if indeed the DNA target was or not being hybridized in the Au electrode.

2.3.2.1. Epifluorescence Microscope analysis for the verification of the optimized biological protocol for E. coli O157:H7

As referred, because suspicions hybridization mass results were recorded by the QCM-D device (assays H_1 to H_5), the verification of results had to be made by the careful analysis of the recorded images taken by the Epifluorescence microscope and treated with the *ImageJ* software.

Table 18 shows the fluorescent fraction area (%), for all the hybridization assays, namely, H_1, H_2, H_3, H_4 and H_5, obtained by the image processing, using *ImageJ 1.45s* software, in order to compare them.

Table 18: Total fluorescent area (% total pixels area) obtained with Epifluorescence Microscope using FITC, TxRd and Cy3-Cy5 filters when analysed in *ImageJ* software in the Hybridization assays.

Assays	FITC mass (% pixels)	TxRd mass (% pixels)	Cy3-Cy5 mass (% pixels)
H_1	0.023	0.016	0.010
H_2	0.013	0.004	0.003
H_3	0.026	0.024	0.007
H_4	0.029	0.037	0.016
H_5	0.022	0.050	0.013

Comparing all the hybridization assays in Table 18, the total fluorescence fraction area (% pixels) for the TxRd fluorochromes, showed an increase of mass hybridized when the hybridization step was made at 30ºC (H_4 and H_5) in the QCM-D device,

compared to the other assays. This results are in good agreement with the published results from Wu and co-workers (2007) study, in where they had an increase of almost 130% in the Hybridization percentage at 30ºC for the DNA target, when compared to the same assay at 20ºC.

In this study, the *ImageJ* software analysis reveals an increase of the detected DNA target of 131% in the H_4 assay, similar to the reported by Wu and co-workers (2007), and an increase of 212% in the H_5 assays, when compared with the H_1 assays.

Regarding the co-immobilization study, similar results were obtained with H_1 and H_3, thus no significant improvement by performing the co-immobilization DNA thiol Probes with the MCH blocking agent, as Carrara and co-workers (2010).

Figure 21 graphically represents the results from Table 17 of the fluorescent mass results for the immobilization (FITC), hybridization (TxRd) and specific hybridization (Cy3-Cy5) percentage area (pixels) in each assay.

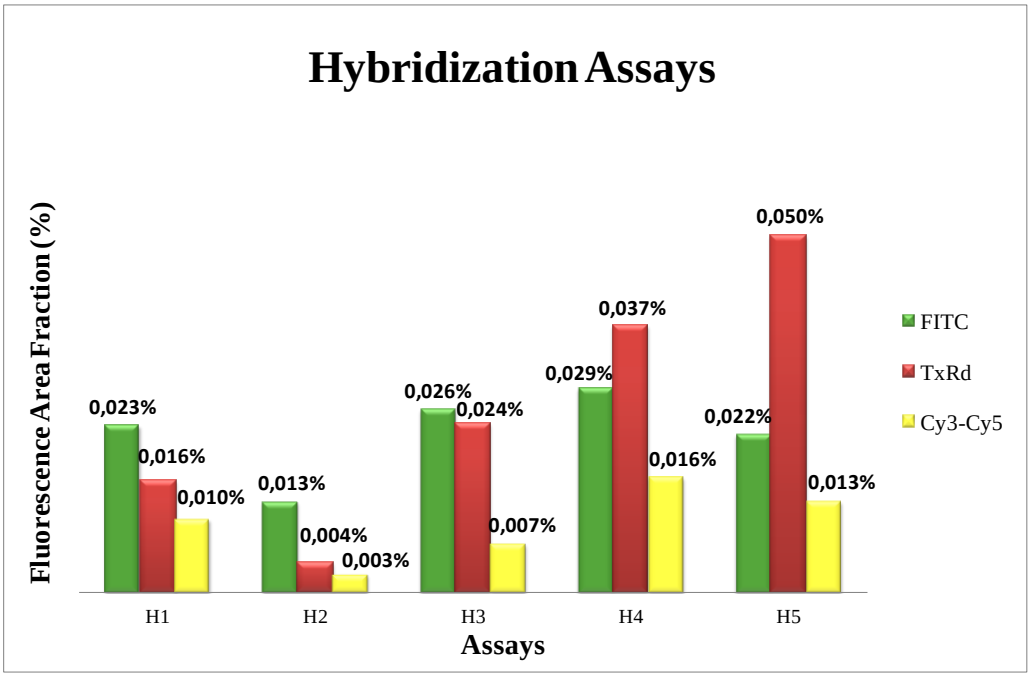

Figure 21: Graphical representation of the total fluorescent area detected, with FITC, TxRd and Cy3-Cy5 filters, by epifluorescence microscopy when analyzed by the *ImageJ* software, in the hybridization assays carried out in the present study.

Figure 21 graphically shows the increase of DNA target hybridized (TxRd) in the H_3, H_4 and H_5 when compared with H_1. However, it also shows that H_5 and H_4 had a higher percentage of DNA target (TxRd) than the one of DNA thiol Probe (FITC), revealing that in these assays, both performed at 30ºC, a very high percentage of *ss*DNA Target were not hybridized with the *ss*DNA thiol Probe, but unspecific immobilized in

the Au electrode. Nevertheless, these assays also showed a higher percentage of Cy3-Cy5 fluorescent area, which represents the specific hybridization mass (in pixels percentage), when compared with the other assays performed at 22ºC.

Figure 22 shows a compilation of the best images performed for each Hybridization assay using the FITC, TxRd and Cy3-Cy5 filters, after the image processing with *ImageJ* software, in order to compare the best assay result.

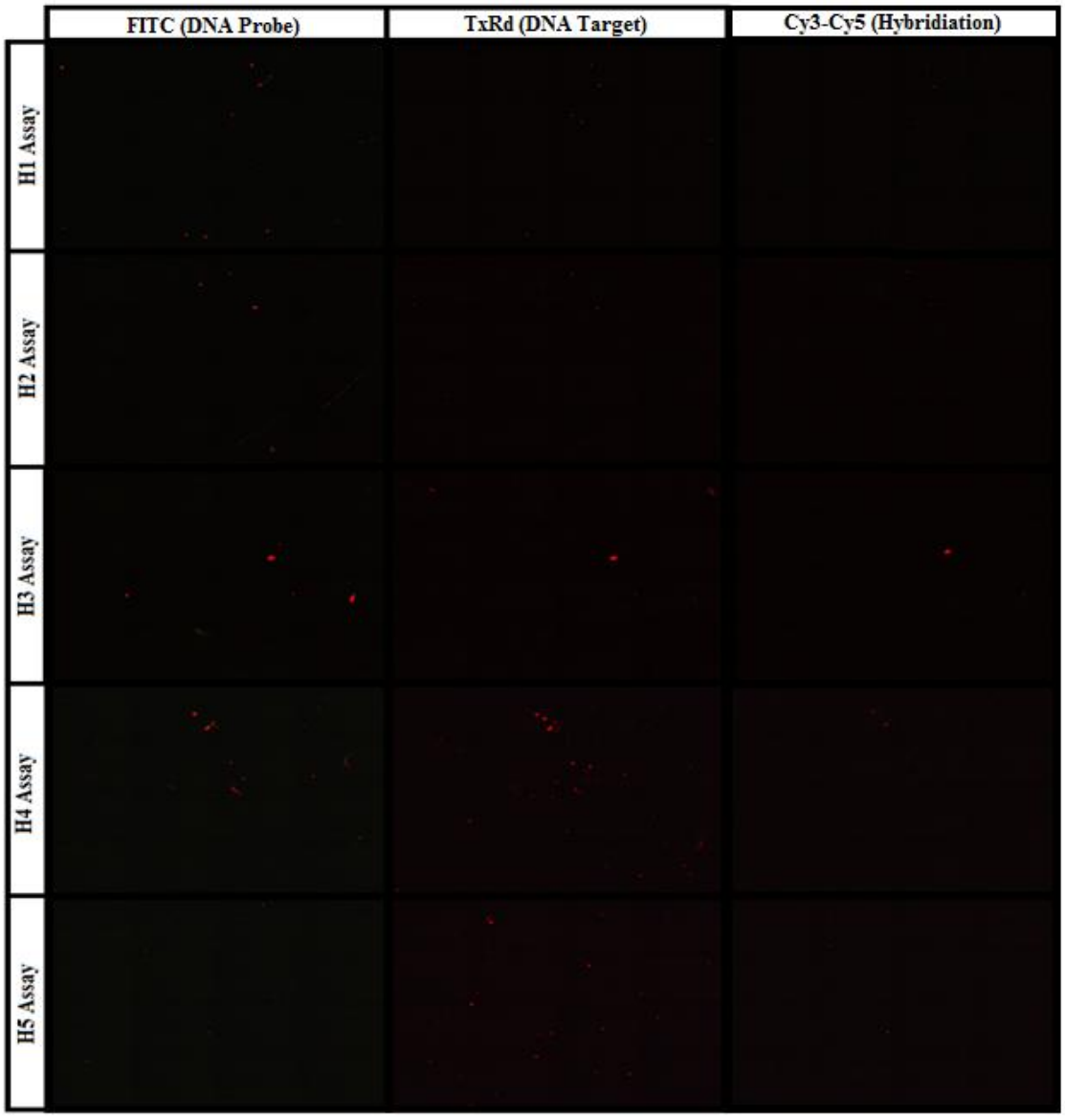

Figure 22: Compilation of the best tests results taken by FITC, TxRd and Cy3-Cy5 filters by Epifluorescence microscope and analysed by ImageJ software.

Both Figure 21 and 22, allow verifying that for all the performed assays, a very poor percentage of fluorescent area was detected for each filter. As referred the use of the NIKON colour camera, which was not the appropriated for the fluorescent images recording, certainly contributed for the poor results.

Figure 22 shows, once more, that assays H_4 and H_5 (hybridization step performed at 30ºC) shown a higher quantity of TxRd fluorescent area recorded, however this quantity is higher that FITC fluorescence area. Meaning that, an increase of temperature can lead to unspecific immobilization of the DNA target in the gold electrode and possible giving false positive results with other non-complementary sequences. For this reason, these assays are for now discarded as optimized ones.

Analysing this compilation of images with the different filters, it can be seen that the quantity of fluorescence recorded is very poor with this camera, even when the images were processing with the software *ImageJ*.

It should be taken into consideration that due to this image processing (segmentation), the fluorescence regions were marked as red colour pixels in all the three filters, but in the images recorded directly from the microscope they had green colour (FITC), red colour (TxRd) and yellow colour (Cy3-Cy5).

Another discussion result that can be made by the analysis of Figure 22, is the poor quantity of fluorescence in the Cy3-Cy5 images, that show much less fluorescent area (%) than when compared with the separated images from FITC and TxRd. This indicates that the average transmission for this dual-filter is too poor at the interesting spectral bands, thus the fluorescent are in reality is possible higher than the one calculated in Table 18.

Based on these results and taking all the precautions, due to the unexpected results in the assays made in QCM-D, and inherent problems that occur with the NIKON camera of the epifluorescence microscope, it is assumed as the best parameters for the hybridization of DNA target (1.00 μM and 60 minutes of incubation time) after the pre-immobilization of the DNA thiol Probe (1.00 μM and 30 minutes of immobilization time), and the MCH blocking agent (1.00 mM and 30 minutes of incubation time) at 22ºC, performed as H_1.

CHAPTER V- CONCLUSIONS AND FUTURE WORK

1. CONCLUSIONS AND FUTURE WORK

In the present work it was proposed as aim, the development and optimization of a biological protocol using a QCM-D device to detect DNA of *E. coli O157:H7* that could be used as a genosensor.

To accomplish this aim, several assays were performed, in order to determinate the best conditions in the steps of DNA Probe immobilization, MCH blocking agent deposition and DNA target hybridization.

Based on the QCM-D mass results from the performed assays and using epifluorescence microscopy to confirm those results, some conclusion can be taken for the present work:

- ✓ Immobilization assays demonstrated that the assay performed as I_5 (1μM of DNA thiol Probe at 30 minutes of immobilization time) accomplished the highest immobilized mass values in the Au electrode, at 22ºC;
- ✓ MCH blocking agent can be prepared with 1x TE buffer instead of the typical Milli-Q water, however, with lower quantity of adsorbed mass for the DNA probe SAM formation on the Au electrode;
- ✓ MCH blocking agent assays, also demonstrated that using the 1x TE buffer, the best condition are made with 1mM of MCH at 30 minutes of incubation time, at 22ºC;
- ✓ Hybridization assays reveal in a first stage, that 1.00 μM of DNA Target in 60 minutes of hybridization time and performed at 22ºC, had a higher hybridized mass than the ones performed with 0.50 μM of DNA Target, for the same time and temperature conditions;
- ✓ In a second stage, the performed Hybridization assays showed an increase of more than 120%, as suggested by the study of Wu and co-workers (2007), in the hybridized DNA target mass when performed with 1.00 μM of DNA Target in 60 minutes of incubation time, at 30ºC; however these results are potentially related to a high quantity of mass not specifically hybridized with the DNA Probes, but in the Au electrode surface;
- ✓ Co-immobilization assays do not reveal an increase of hybridized DNA target mass, as Carrara and co-workers (2010) suggested, using the present study conditions.

This study also showed that the epifluorescence microscopy, using image processing with *ImageJ* software, was a very helpful and useful tool for the verification and optimization of the final biological protocol.

Based on the results obtained under the herein used conditions, it was concluded that the best conditions for *E. coli O157:H7* DNA detection using the QCM-D *Q-Sense E1* were obtained with the optimized biological protocol performed at 22ºC with: immobilization of 1.00 μM of DNA Probe at 30 minutes of incubation time, followed by 1.00 mM of MCH blocking agent during 30 minutes of incubation time and hybridized with 1.00 μM of DNA Target at 60 minutes of incubation time.

The present study also revealed some weaknesses in the development of this biological protocol.

The results appears to demonstrate that the 1x TE buffer suggested by the *Sigma-Aldrich®*, was not the best buffer to be used in the QCM-D *E1* device for DNA detection, due to the instable baselines sometimes recorded, as well as, the weak Frequency signal (Hz) recorded, when compared with other published studies, that used PBS buffer with a similar pH.

Probably for this reason, the sensitive QCM-D recorded, for several times, negative and/or variable DNA mass results, thus several replicated test assays had to be additionally made, in order to obtain three valid results. Moreover, the obtained results frequently were not in agreement with the epifluorescence microscopy images.

Based on the results herein obtained, further studies it is suggested to use a PBS buffer (possibly with pH around 8.0 and molar concentration around 1M), as Choo and co-workers (2004), and replicate this optimized protocol, with the same optimized concentration, time and temperatures parameters, in order to prove if the PBS buffer is more stable and reproducible for the DNA detection on QCM-D than 1x TE buffer, as the results of other published studies appear to suggest.

Another interesting future study would be the use of the proposed methodology for futher applications, namely the development of an electrochemical DNA biosensor for rapid DNA analysis and pathogen detection.

The present work was submitted and accepted as a poster presentation to the *11º Encontro da Química dos Alimentos – Qualidade dos Alimentos: Novos Desafios* in

Bragança (Portugal) on September, 2012. The abstract submission is presented in *Appendix VIII*.

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APPENDIX

APPENDIX I – BUFFER 1X TE (10MM TRIS-HCL + 1MM EDTA, PH 7.5-8.0)

A- <u>1M Tris (Tris(hydroxymethyl) aminomethane), FW=121.4g/mol, Vf=500ml</u>

i. 60.57g of Trizma was weighed and diluted on 300ml of Milli-Q water (18.2 MΩcm; Milipore, Tokyo);
ii. The solution was diluted with the aid of an electrode and a magnetic plate on a vertical laminar flow workstation;
iii. pH was measured with a digital pH meter until pH10;
iv. HCl at 30% was added in order to lower the pH from 10 to 7.5 on a continuous reading with a digital pH meter;
v. The solution was filed on a volumetric flask was to 500 ml with Milli-Q water;
vi. Mixed 3-5 times;
vii. Identified with date, solution name and pH.

B- <u>0.5M EDTA (Diaminoethane tetraacetic acid), FW=372.2 g/mol, Vf=100ml</u>

i. 18.6g of EDTA was weighed and diluted with 70 ml of Milli-Q water;
ii. The solution was diluted with the aid of an electrode and a magnetic plate on a vertical laminar flow workstation;
iii. pH was measured with a digital pH meter until pH 6.5;
iv. A solid NaOH was added (1 grain at time, to don't overheat the acidic solution), until obtained a solid reaction solution (arround pH 7.8);
v. Drops of 10M NAOH was added until the solution passed from a solid to a transparent liquid solution;
vi. pH was confirmed with a digital pH meter (about pH 8)
vii. The solution was filed on a volumetric flask was to 100 ml with Milli-Q water;
viii. Mixed 3-5 times;
ix. Identified with date, solution name and pH.

C- <u>Buffer 1x TE, Vf=1L</u>

i. Mixed 10 ml of 1M Tris (**solution A**) and 2ml of EDTA 0.5M (**solution B**);
ii. Added 988ml of Milli-Q water on a bottle of 1L;

iii. Mixed 3-5 times;

iv. Identified with date, solution name and pH;

v. The buffer 1x TE solution was autoclaved during 20 minutes at 121ºC.

APPENDIX II – PHOSPHATE BUFFERED SALINE (PBS), PH 8.3-8.5

A- Prepare PBS from pH 7.4 to 8.3-8, Vf=1000ml

i. The content of a PBS sachet (*Sigma-Aldrich, Nº. P5368, para 0.01M de PBS a pH7.4 a 25ºC*) was dissolved with 800 ml of Milli-Q water (18.2 MΩcm; Milipore, Tokyo), on a vertical laminar flow workstation, aid by an electrode and a magnetic plate;
ii. pH was measured with a digital pH meter in continuous flow;
iii. A NaOH 1M was added in order to raise the pH from 7.4 to 8.35;
iv. The solution was filed on a volumetric flask was to 1000 ml with Milli-Q water;
v. Mixed 3-5 times;
vi. Identified with date, solution name and pH.

APPENDIX III- 3M DE SODIUM ACETATE SOLUTION

A- Preparation of a Sodium Acetate solution from pH 7.0-8.0 to 5.2, FW= 82,03g/L, Vf=500ml

i. 123,045g of Anidric Sodium Acetate (*Sigma-Aldrich, Nº. S2889)* was weighed and diluted with 300 ml Milli-Q water (18.2 MΩcm; Milipore, Tokyo) with an aid of a magnetic plate;
ii. pH was measured on a continuous flow by a pH meter until pH 7.0-8.0;
iii. HCl at 30% was used in order to lower the pH from 7.0-8.0 to 5.2;
iv. The solution was filed on a volumetric flask was to 500 ml with Milli-Q water;
v. Mixed 3-5 times;
vi. Identified with date, solution name and pH.

APPENDIX IV- DETERMINATION OF MASS DNA THIOL PROBE CONCENTRATION BY SPECTROPHOTOMETER UV-VIS ANALYSIS

The following protocol adapted from Geneworks (2011) was carried out:

- An aliquot of DNA thiol probe was diluted and resuspended in 1x TE solution to a final volume of 1000 µL;
- Was calculated the dilution factor (for instance, for a dilution of 50 µL of DNA thiol probe, the dilution factor will be, 1000/50=20);
- The vortex was used to obtain a homogeneous mixture solution;
- The absorbance was read on 260 nm peak.

(NOTE: Was blank it was used the 1x TE buffer solution in the spectrophotometer)

Data:

- **Dilution factor:** 10 µl DNA thiol Probe/ 999 µl 1xTE buffer = 100.9
- $\mathbf{A_{260\ E.\ coli\ DNA\ thiol\ Probe}}$**:** 0.228 Abs
- **l=** 1 cm of quartz curette
- $\mathbf{\varepsilon_{260}}$**=** ~33 for *ss*DNA
- $\mathbf{V_{final\ DNA\ thiol\ Probe}}$**=** 600 µl
- $\mathbf{MW_{DNA\ thiol\ Probe}}$**=** 7215

1º Determination of the DNA thiol Probe concentration by Spectrophotometer analysis:

$$Stock\ Conc.\left(\frac{\mu g}{\mu l}\right) = \frac{A_{260}}{l \times \varepsilon_{260}} \times Dilution\ factor \qquad [4]$$

Results:

Stock Concentration= 0.697 µg/µl

2º Determination of the Molarity concentration:

If:

1M ---------- 7.215 g/ml

x ---------- $0.697x10^{-3}$ g/ml

Where:

$x= 9.55x10^{-5}$ M = 96.60 μM

3º Recovered DNA thiol Probe mass

$m_{recovered}$= 600 μl x 0.697 μg/μl = 418.20 μg

$m_{total\ from\ Sigma\text{-}Aldrich}$= 684.40 μg

$ml_{ost\ in\ the\ ethanol\ protocol}$= 684.40 – 418.20 = 266.20 μg

Representing a 61.10% of DNA thiol Probe mass recovered

APPENDIX V- DETERMINATION OF THE DESIRE CONCENTRATIONS (MM) TO ALIQUOT THE DNA THIOL PROBES

1ºAliquots with 0.25µM in 250µl:

C_{final}= 0.25µM
$C_{initial}$= 96.60 µM
V_{final}= 250 µl
$V_{initial}$= ?

Using the following equation:

$$Ci \times Vi = Cf \times Vf$$

Where:

$V_{initial}$= 0.65 µl of DNA thiol Probe in Stock

In order to obtain the final aliquots, it was added $V_{final} - V_{initial}$ of 1xTE buffer.

2ºAliquots with 0.50µM in 250µl:

C_{final}= 0.50µM

$C_{initial}$= 96.60 µM
V_{final}= 250 µl
$V_{initial}$= ?

Using the equation:

$$Ci \times Vi = Cf \times Vf$$

Where:

$V_{initial}$= 1.29 µl of DNA thiol Probe in Stock

In order to obtain the final aliquots, it was added $V_{final} - V_{initial}$ of 1xTE buffer.

3ºAliquots with 1.00µM in 250µl:

C_{final}= 1.00 µM
$C_{initial}$= 96.60 µM
V_{final}= 250 µl
$V_{initial}$= ?

Using the equation:

$$Ci \times Vi = Cf \times Vf$$

Where:

$V_{initial}$= 2.59 µl of DNA thiol Probe in Stock

In order to obtain the final aliquots, it was added $V_{final} - V_{initial}$ of 1xTE buffer.

2ºAliquots with 2.00 µM in 250µl:

C_{final}= 2.00 µM
$C_{initial}$= 96.60 µM
V_{final}= 250 µl
$V_{initial}$= ?

Using the equation:

$$Ci \times Vi = Cf \times Vf$$

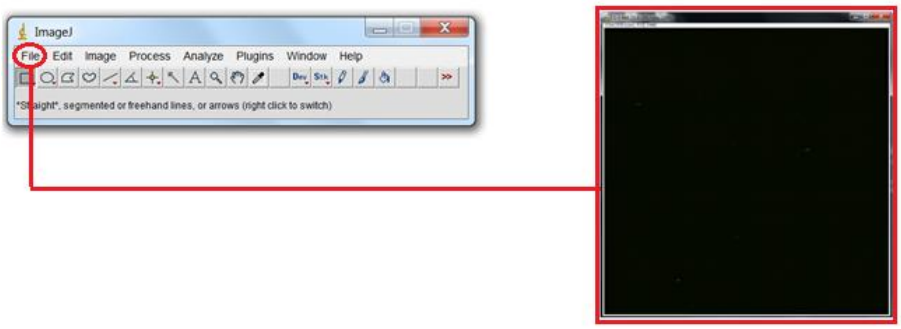
Where:

$V_{initial}$= 5.18 µl of DNA thiol Probe in Stock

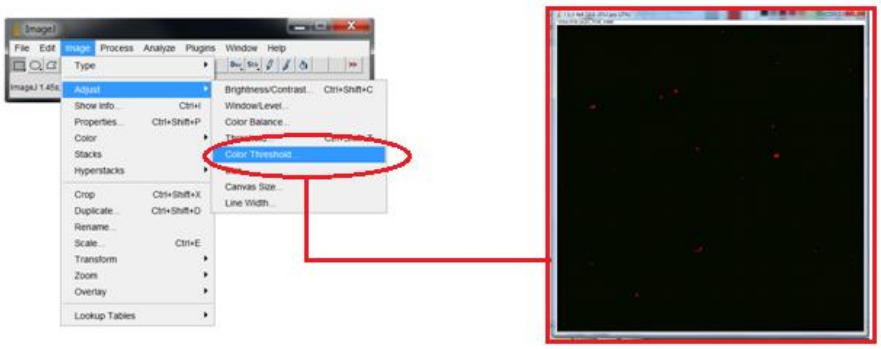
In order to obtain the final aliquots, it was added $V_{final} - V_{initial}$ of 1xTE buffer.

APPENDIX VI- EXAMPLE OF THE DETERMINATION OF FLUORESCENCE MASS IN THE IMMOBILIZATION AND/OR HYBRIDIZATION ASSAYS BY *IMAGEJ 1.45S* SOFTWARE

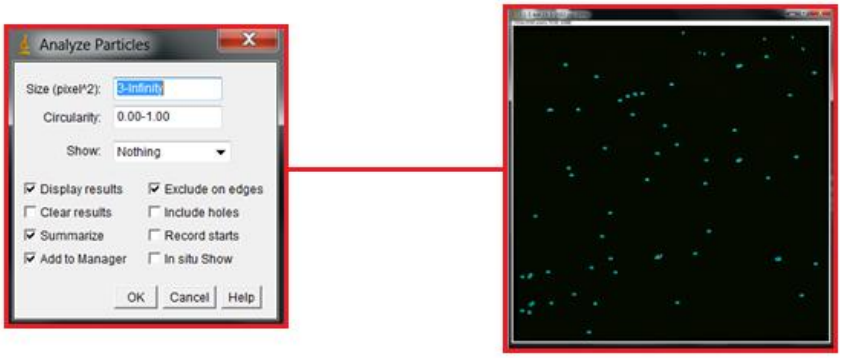
1. Epifluorescence images where imported to the *ImageJ 1.45s* software



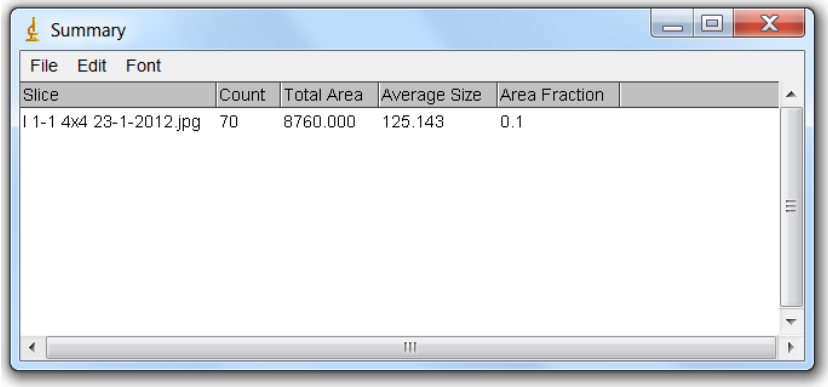
2. In the command *Image* → *Adjust* → *Color Threshold* the *Hue*, *Saturation* and *Brightness* variants were adjusted to give the best contrast of the fluorescence mass signal;



3. In the command *Analyze* →*Analyze particles* the *Size* (pixel^2) was selected between 3 and Infinity (eliminating possible background pixels in the image);



4. The display summary results with *Count Particles*, *Total Area*, *Average Size* and *Area Fraction* was saved for each assay and analysed in Microsoft Office Excel 2007.



APPENDIX VII – DNA THIOL PROBES MASS RESULTS BY QCM-D DEVICE

Time Incubation (min)

Concentration (µM)	30		60	
	Assays	**Mass (ng/cm^2)**	**Assays**	**Mass (ng/cm^2)**
0.25	$I_{1.1}$	8.04	$I_{2.1}$	-9.48
	$I_{1.2}$	24.38	$I_{2.2}$	-33.48
	$I_{1.3}$	0.74	$I_{2.3}$	11.74
	$I_{1.4}$	6.38	$I_{2.4}$	9.93
	$I_{1.5}$	71.04	$I_{2.5}$	29.74
			$I_{2.6}$	36.76
			$I_{2.7}$	-4.14
			$I_{2.8}$	29.99
			$I_{2.9}$	12.80
0.5	$I_{3.1}$	8.68	$I_{4.1}$	20.58
	$I_{3.2}$	-30.90	$I_{4.2}$	13.58
	$I_{3.3}$	-11.64	$I_{4.3}$	-8.48
	$I_{3.4}$	29.53	$I_{4.4}$	-9.63
	$I_{3.5}$	-21.33	$I_{4.5}$	19.49
	$I_{3.6}$	9.72	$I_{4.6}$	10.60
1.0	$I_{5.1}$	25.14	$I_{6.1}$	26.42
	$I_{5.2}$	-24.92	$I_{6.2}$	-23.68
	$I_{5.3}$	18.83	$I_{6.3}$	6.26
	$I_{5.4}$	10.20	$I_{6.4}$	2.57
			$I_{6.5}$	19.49
			$I_{6.6}$	10.60
2.0	$I_{7.1}$	51.31	$I_{8.1}$	-42.47
	$I_{7.2}$	-7.15	$I_{8.2}$	8.90
	$I_{7.3}$	6.47	$I_{8.3}$	-19.35
	$I_{7.4}$	-9.41	$I_{8.4}$	29.61
	$I_{7.5}$	20.43	$I_{8.5}$	14.19
	$I_{7.6}$	4.25		

APPENDIX VIII- PUBLICATIONS

11º Encontro de Química dos Alimentos

Detecting *Escherichia coli* O157:H7 by Quartz Crystal Microbalance with Dissipation (QCM-D)

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Escherichia coli O157:H7 is a foodborne pathogen classified as an Enterohemorrhagic *E. coli* being associated to foodborne outbreaks with high mortality. Since the traditional methods for its detection are often time-consuming, there is a need to develop new techniques that allow a rapid, simple, reliable, specific and sensitive detection for a quick and effective medical intervention, as well as to ensure food safety. In the last years different approaches have been suggested for this purpose including the use of biosensors based on different bioreceptors such as enzymes, antibodies and DNA. Recently, there has been a growing interest regarding DNA biosensors due to advantages such as DNA high specificity and stability and the possibility of developing devices that can be reusable after thermal melting of the DNA duplex [1].

Since these genosensors use immobilized DNA single strands to detect the complementary sequence by hybridization, it is very important to optimize the conditions used during probe immobilization and target hybridization. In this work, a DNA piezoelectric biosensing method for *E.coli* O157:H7 DNA detection was developed and optimized using a QCM-D to evaluate the immobilization/hybridization mass phenomena. A 21-mer oligonucleotide sequence (probe) and its complementary strain (DNA target) were selected from the *eae* gene of *E. coli* O157:H7. DNA probe was modified with a C6 alkanethiol group to improve immobilization by Self-Assembled Monolayer on the gold electrode surface. To prevent non-specific adsorption of DNA during the immobilization and hybridization steps, 6-mercapto-1hexanol (MCH) was used as a blocking agent. Different parameters such as probe concentration (0.25, 0.5, 1.0, 2.0µM) and incubation time (30 and 60min) on the immobilization step, MCH concentration (0.5 and 1.0µM) and incubation time (30 and 60min), target concentration (0.5 and 1.0mM) and hybridization temperature (22 and 30ºC) were tested. Co-immobilization assays using a mixture of DNA thiol Probe and MCH, were also performed at 22 and 30ºC. The best results regarding immobilization on the gold electrode at 22ºC were obtained using 1µM of DNA probe and 30min of incubation. Co-immobilization of DNA thiol probe and MCH did not revealed a significant improvement. When using 1 µM target DNA, higher hybridization efficiency was observed at 30ºC. Optical validation of the developed protocol was achieved by epifluorescence microscopy, used to identify different fluorescent labels on the DNA thiol probe and target. The proposed methodology can potentially be used for further applications, namely the development of an electrochemical DNA biosensor for rapid DNA analysis and pathogen detection.

[1] FRR Teles, LP Fonseca, Talanta, 2008, 77, 606-623.