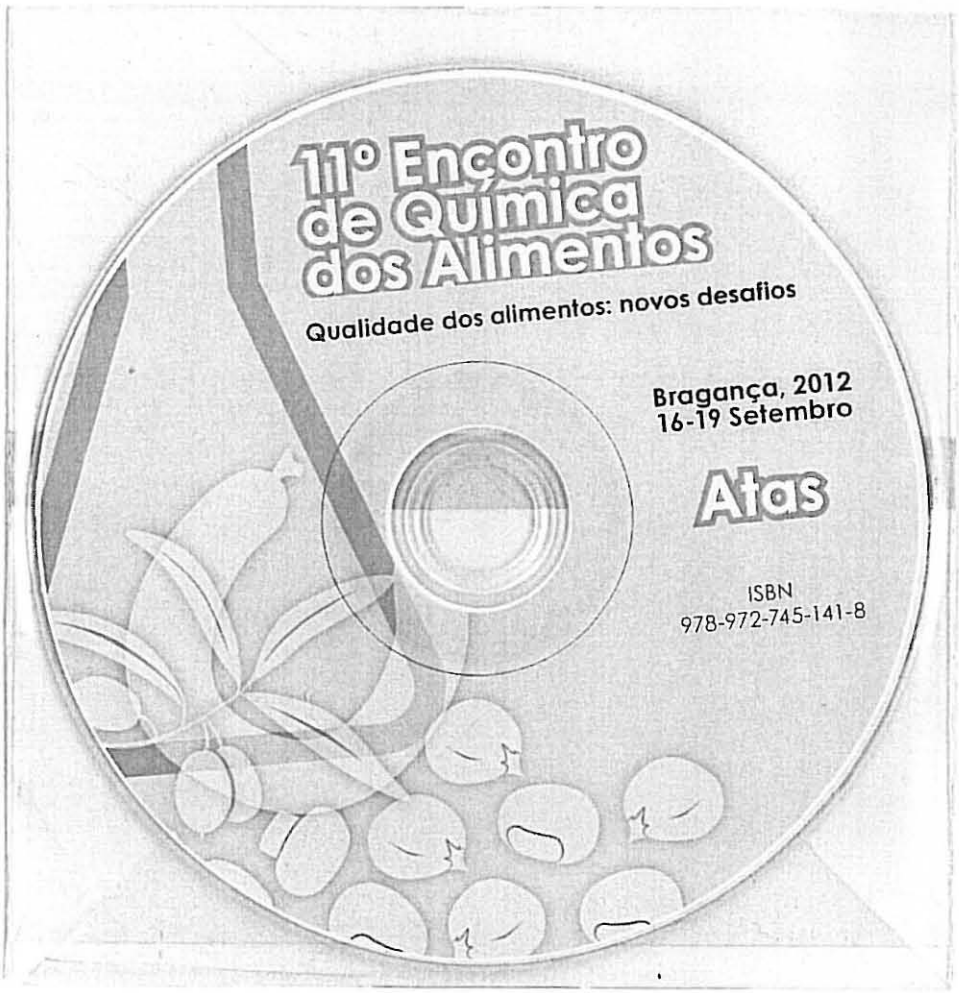
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Detecting *Escherichia coli* O157:H7 by Quartz Crystal Microbalance with Dissipation (QCM-D)

Raquel O. Rodrigues[a,b]*, *Eva Pérez-Lorenzo*[b], *Sergio Arana*[b], *Joana S. Amaral*[a,c], *Pedro S. Rodrigues*[a], *Maite Mujika*[b]

[a]ESTiG/Polytechnic Institute of Bragança, Bragança, Portugal
[b]CEIT and Tecnun (University of Navarra), San Sebastián, Spain
[c]REQUIMTE, Pharmacy Faculty, University of Porto, Portugal
*oliraquel.rodrigues@gmail.com

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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 *E. coli* O157:H7 using a Quartz Crystal Microbalance with Dissipation (QCM-D), to be applied as a genosensor based on the evaluation of the immobilization/hybridization mass phenomena. Since 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 study, several parameters (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. Additionally, 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.

1. INTRODUCTION

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 *E. coli O157* strains causing about 36% of these infections. The majority of conventional methods to detect foodborne pathogens are often time-consuming and, additionally, require intricate specific instrumentation, highly trained operators and cannot be used *in situ* at low cost. Thus, there is a need for new technologies that allow a rapid, reliable, simple, specific and sensitive detection of the microorganism, for a quick and effective medical intervention, as well as a rapid eradication of the focus infection diseases [1]. In the last years, there has been much research activity in the area of biosensors development for microorganisms' detection, with different approaches being proposed. Among these, DNA biosensors have

recently emerged as one of the most promising techniques for foodborne pathogens detection, mainly due to its low cost and speed of 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. The QCM-D has been recently used, for the detection of the immobilization/hybridization mass phenomena, due to its sensibility, robustness and reliability. The QCM is a very sensitive sensor that detects mass changes based on the piezoelectric effect. This equipment uses a quartz crystal and a gold disk where an electric field is applied (metallic electrodes). The mass deposition on the gold electrode causes inertial changes on the crystal surface leading to oscillation frequency shifts. The oscillation frequency changes in the quartz crystal is related to the mass changes using the Sauerbrey equation (Equation 1), where Δf: frequency change (Hz); $f0$: crystal piezoelectric oscillation overtone frequency (Hz); A: piezoelectric active crystal area (m^2); Δm: mass change on the metallic material surface (g); ρq: density of quartz (ρq=2.648 g/cm^3); μq: shear modulus of quartz for AT-cut crystal (μq=2.947x10^{11}g/cm.s^2) [2].

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

Using the QCM device and the Sauerbrey equation it is possible to detect in real-time, the increase of mass bound to the electrode/quartz crystal surface, thus allowing monitoring DNA immobilization and subsequently the sequence-specific DNA hybridization.

2. MATERIALS AND METHODS

2.1 Oligonucleotides

The two complementary oligonucleotides were selected from *eae* gene of *E. coli* O157:H7 which encodes intimin adhesion protein, essential to the bacteria virulence. The DNA Probe was modified with a C6 alkanethiol (ThiC6) in the 5'-end to improve the immobilization and Fluorescein (FITC) in the 3'-end to identify the DNA probes by Epifluorescence microscopy, after the immobilization step. The fluorochrome Texas red (TxRd) was added in the 5'-end of the complementary target oligonucleotide to identify hybridization by Epifluorescence microscopy. The probes were acquired to Sigma-Aldrich and are represented in table 1.

Table 1. 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'

2.2 QCM-D assays

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)) was cleaned using a BioForce ProCleanerTM Ultra-Violet and Ozone (UV/Ozone) chamber and a 5:1:1 solution of Milli-Q water/Ammonia/ Hydrogen Peroxide 25%.

2.2.1. *Probe immobilization with self-assembled monolayer (SAM) formation:* Eight immobilization assays (*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 min) were performed.

2.2.2. 6-mercapto-1hexanol (*MCH) blocking agent during SAM formation:* The MCH blocking agent solution was prepared in Milli-Q water and 1x TE buffer. Four blocking agent assays (*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.

2.2.1. *Hybridization assays:* hybridization assays (*H*) were carried out, using different target DNA concentration (0.50 and 1.00 µM) and hybridization temperature (22 and 30ºC). Two co-immobilization assays (DNA probe + MCH) were also performed at 22ºC and 30ºC.

2.3 Epifluorescence microscopy and digital image processing

An inverted microscope system (NIKON®, Eclipse Ti) with epifluorescence, coupled to a HAMAMATSU® digital camera or to a NIKON® colour camera was used to observe the Au electrode. Three types of filters were used: *FITC*, *TxRd* and *Cy3-Cy5* (which enabled to visualize both fluorochromes, FITC and TxRd, after hybridization). The obtained images were submitted to a segmentation process using the *ImageJ* 1.45s software and afterwards the segmented fluorescence regions were filtered and the fraction (%) of fluorescent area calculated (Area Fraction) aiming to infer a correlation with the immobilized mass.

3. RESULTS AND DISCUSSION

Figure 1 shows the obtained results of an immobilization assay with the real-time recording of the Frequency variation (Hz) represented in blue colour and the Dissipation ($1x10^{-6}$) represented in orange colour, recorded with the 9th overtone with the QCM-D E1 device.

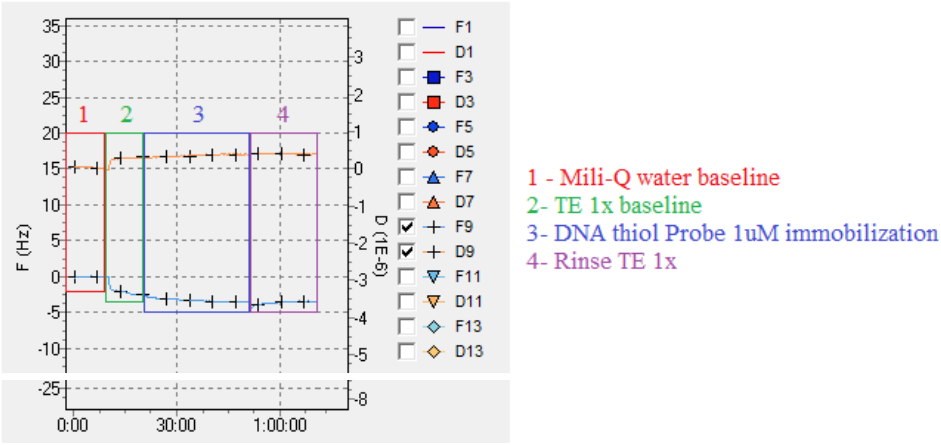

Figure 1. Immobilization assay performed at 22ºC using 1.00 µM DNA thiol Probe and 30min incubation time (I_5) recorded using the 9th overtone of the QCM-D E1 device.

The results obtained in each QCM-D assay were traduced to mass deposition values after applying the Sauerbrey equation allowing to conclude that the best results regarding immobilization on the gold electrode at 22ºC were obtained using 1µM of DNA probe and 30min of incubation (Fig 2A). Figure 2B evidences that higher mass of blocking agent deposition was obtained using 1mM MCH and 30 min incubation time.

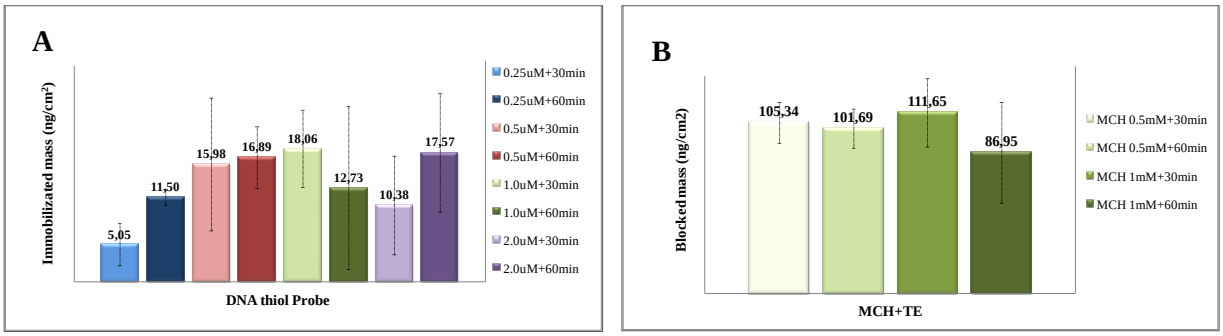

Figure 2. **A:** Average mass deposition and standard deviation obtained for the DNA thiol probe immobilization assays with different concentrations and incubation time; **B:** Average mass deposition and standard deviation regarding deposition of MCH blocking agent prepared in 1xTE buffer.

Regarding the five hybridization assays performed, no conclusive results were obtained using the QCM-D device, thus the electrodes were observed by epifluorescence microscopy and the images submitted to digital image processing. The fluorescent Area Fraction calculated is shown in Figure 3. It can be observed an increase of hybridized mass (TxRd) when the hybridization step was made at 30ºC (H4 and H5). However both these assays had a much higher percentage of DNA target (TxRd) than the one of Probe (FITC) which suggests unspecific DNA immobilization in the gold electrode. Considering the higher Cy3-Cy5 %, the best conditions seem to be the ones used in H1 assay at 22ºC (1.00 µM DNA Probe/30 min; 1.00 mM MCH blocking agent/30 min; hybridization using 1.00 µM DNA target/ 60 min).

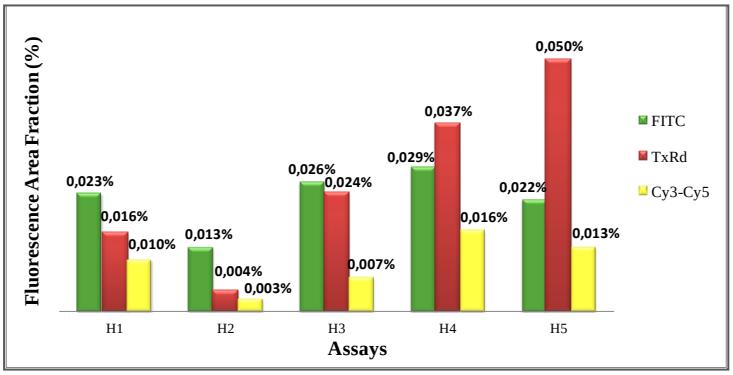

Figure 3. Total fluorescent Area Fraction obtained after segmentation with ImageJ software for the hybridization assays images using *FITC*, *TxRd* and *Cy3-Cy5* filters in the epifluorescence microscope

4. CONCLUSION

The proposed methodology allowed the specific detection of *E. coli* O157:H7 DNA and can potentially be used for further applications, namely the development of an electrochemical DNA biosensor for rapid DNA analysis and pathogen detection.

References

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