

UNIVERSITY OF SÃO PAULO
FFCLRP - DEPARTMENT OF PHYSICS
POSTGRADUATE IN PHYSICS APPLIED TO MEDICINE AND BIOLOGY

**Detecção de alterações relacionadas ao câncer de
mama através de sensores de carga ou de massa
acoplados a monocamadas de DNA**

(Detection of changes related with breast cancer using
charge sensors or mass sensors coupled to DNA monolayers)

MARINA RIBEIRO BATISTUTI

Thesis submitted to Faculty of Philosophy,
Sciences and Literature of University of São
Paulo, as part of the requirements for the
degree of Doctor in Science. Area: Physics
applied to Medicine and Biology.

Ribeirão Preto - SP

2017

MARINA RIBEIRO BATISTUTI

**Detecção de alterações relacionadas ao câncer de
mama através de sensores de carga ou de massa
acoplados a monocamadas de DNA**

Thesis submitted to Faculty of Philosophy,
Sciences and Literature of University of São
Paulo, as part of the requirements for the
degree of Doctor in Science.

Concentration area:

Physics applied to Medicine and Biology.

Supervisor:

Marcelo Mulato.

Co-supervisor:

Paulo Roberto Bueno.

Versão corrigida

Versão original disponível na FFCLRP-

Ribeirão Preto - SP

2017

I authorize to copy and disclosure total or partial of this document by any conventional or electronic media, for the purpose of study and research, as long as cited the source.

FICHA CATALOGRÁFICA

Batistuti, Marina Ribeiro

Detecção de alterações relacionadas ao câncer de mama através de sensores de carga ou de massa acoplados a monocamadas de DNA / Marina Ribeiro Batistuti; supervisor Marcelo Mulato. Ribeirão Preto - SP, 2017.

122 f.:il.

Thesis (Ph.D. - Program of Postgraduate in Physics applied to Medicine and Biology) - Faculty of Philosophy, Sciences and Literature da University of São Paulo, 2017.

1. Electrochemical. 2. QCM. 3. DNA. 4. Aptamer.

Name: BATISTUTI, Marina Ribeiro

Title: Detecção de alterações relacionadas ao câncer de mama através de sensores de carga ou de massa acoplados a monocamadas de DNA

Thesis submitted to Faculty of Philosophy,
Sciences and Literature of University of São
Paulo, as part of the requirements for the degree
of Doctor in Science.

Approved in: ____/____/____.

Examiners

Prof. Dr. : _____ Institution: _____

Judgment: _____ Signature: _____

Prof. Dr. : _____ Institution: _____

Judgment: _____ Signature: _____

Prof. Dr. : _____ Institution: _____

Judgment: _____ Signature: _____

Prof. Dr. : _____ Institution: _____

Judgment: _____ Signature: _____

Prof. Dr. : _____ Institution: _____

Judgment: _____ Signature: _____

Dedicated in loving memory of my friend

Natalia Biziak

who supported me and this project since the beginning.

Your absence will always be felt.

Dedicado com carinho a memória da minha amiga

Natalia Biziak

que me apoiou e apoiou este projeto desde o início.

Sua ausência será sempre sentida.

ACKNOWLEDGMENT

This project received the necessary help and support from different areas which I will be eternally grateful.

I am deeply grateful to my supervisor Professor Marcelo Mulato, who supported this work, trusted me to develop it and encouraged me to go further. I also express my gratitude to my co-supervisor Professor Paulo Roberto Bueno, for his support and advice.

I express my warmest gratitude to Professor Pedro Estrela from University of Bath for receiving me on his lab. His guidance, insightful comments and suggestions were indispensable for this thesis. I am deeply grateful to Professor Michael Lodomery for introduced me on molecular biology and always show his passion on this subject.

All my friends and colleagues from the laboratory in Ribeirão Preto, especially Jessica Colnaghi Fernandes, Haroldo Lima Pimentel Cravo, Guilherme de Oliveria Silva, Raphael Aparecido Nascimento, Hugo José Dias Mello, Ricardo França Rocha and Fernando Mano for their help and friendship. I would like to express my gratitude to Bassam Junior, Stephanie Lisboa, Joyce Moraes and Daísy Camargo Ferreira. Thank you for allowing me to share with you the little that I know.

My friends and colleagues in the laboratory in Araraquara, especially Adriano Santos, Tiago Azevedo and Flávio Bedatty Fernandes. Thank you for all patience and help. From University of the West of England, I would like to express my gratitude to Samantha Jumbe, Elizabeth Bowler and Chigeru Wodi for all the patience and time spent with me.

From University of Bath, my intellectual debt is to Pawan Jolly, who guided me over the months there, trusted me and shared all his knowledge. I would like

to offer my special thanks to Pavel Zhuravski, Serife Ustuner, Nikhil Bhalla, Nello Formisano, Anna Miodek, Jules Hammond, Jahnavi Jha and Sunil Arya. They made the everyday life of PhD in Bath such enjoyable experience.

I would also like to express my gratitude to FAPESP for all financial support in Brazil (2013/26133-7) and in UK (2015/14403-5 and 2015/14404-1).

I owe my deepest gratitude to my parents Paschoal and Cristina, my brothers Guilherme e Nicolas and my fiancé David, who continuous supported me and encouraged over this years and made it possible. Words will never be enough to say thank you!

Finally, to my countless friends that made my life so much happier and will be impossible to name them here!

To God, all praise, honor and glories.

ABSTRACT

BATISTUTI, M. R. **Detection of changes related to breast cancer using charge sensors or mass sensors coupled to DNA monolayers.** 2017. 122 f. Thesis (Ph.D. - Postgraduate program in Physics applied to Medicine and Biology) - Faculty of Philosophy, Sciences and Literature, University of São Paulo, Ribeirão Preto - SP, 2017.

The electrochemical biosensor has been extensively used due to its capacity for rapid and accurate detection of a wide variety of target molecules or biomarkers. DNA hybridization sensors are based on the increase of negative charge on the electrode surface after the DNA target hybridize to the immobilized probes. The development of this platform requires first an understanding of the immobilization process and optimization of surface probe density. In this thesis the electron transfer is investigated on a label-free DNA hybridization detection by its intrinsic charge. The investigation using different immobilization buffers shows a strong dependence on their composition and concentration, and also the influence of the probe and spacer co-immobilized to obtain an organized and compact self-assembled monolayer. The probe density is determined using the chronocoulometry method with hexaammineruthenium (III) chloride, where the value is calculated from the number of cationic redox molecules electrostatically associated with the anionic DNA backbone and presented a linear relationship between thiol molar fraction and probe density from 2 to 5×10^{12} molecules/cm². The effect of hybridization was determined using electrochemical impedance spectroscopy using negatively charged ferri/ferrocyanide redox couple in solution. After probe surface density optimization, the maximum shift of charge transfer resistance (20%) upon 1 μ M complementary sequence was obtained with around 25% probe fraction immobilized

on surface. This electrochemical platform developed was able to detected 100 pM of target sequence and distinguish mismatched sequences. The limit of detection is higher when compared to the literature, however, this system can be further improved by amplifying the signal. The same platform is reproduced in the quartz crystal microbalance system and with field-effect transistor, comparing the different detections. The same platform is tested using two different HER2 aptamer sequences. Biological aspects are explored for a better understanding of the system.

Key-words: 1. Electrochemical. 2. QCM. 3. DNA. 4. Aptamer.

LIST OF FIGURES

2.1	Electrochemical DNA biosensor system. In (a) an illustration with the all the elements and processes. In (b) the equivalent electric circuit of the system.	6
2.2	Illustration of double layer region of an electrochemical interface according to Helmholtz in (a), Gouy-Chapmann in (b) and the Gouy-Chapman-Stern model in (c).	7
2.3	Illustration of the electrochemical cell. In (a) the correct position between the reference, working and counter electrodes. In (b) the equivalent circuit to describe the cell with the capacitance at the working electrode (C_w) and the solution resistance (R_s).	8
2.4	Illustration of the mass transport from the bulk to the electrode surface, electron transfer at the electrode surface and other chemical reactions following the electron transfer.	8
2.5	Illustration of the cyclic voltammetry process. In (a) the potential simulation and in (b) the current response.	10
2.6	Illustration of the cronocoulometry from the cronoamperometry. In (a) the step potential from E_1 to E_2 over the time. In (b) the current response over the time and in (c) the charge vs. $\text{time}^{1/2}$ to extract the surface density information.	12
2.7	Illustration of the potential and current waves. In black a period of the potential (E) wave and, in blue, the a period of the current wave with a shift ϕ from the E	14
2.8	Nyquist example of a RC circuit in serie.	16
2.9	Nyquist example of a RC circuit in parallel.	17

2.10	The electrochemical interface model. In (a) the Randles circuit and the correspond Nyquist curve in (b). R_e correspond to electrolyte resistance or solution resistance, Cdl the double-layer capacitance and R_{ct} the charge transfer resistance related to the electrode process.	18
2.11	Illustration of a QCM system (a) with the correspond electric circuit (b).	20
2.12	Comparison between the classical EGFET system built with a MOSFET (a) and an alternative EGFET systems built with an instrumental amplifier (b).	22
3.1	Illustration of ssDNA immobilization on a gold surface (a), followed by a MCH immobilization (b) to built a self-assembled monolayer (SAM) and the hibridization event (c).	25
3.2	Cyclic voltammetry of the cleanning procedures. First, in (a), the dessorption in 0.5 NaOH follow by the eletrochemical cleanning (b) in 0.5 M H_2SO_4 . Both using Hg/Hg_2SO_4 as reference electrode.	27
3.3	Cyclic voltametry of the electrochemical cleanning procedure from -0.5 to 1.1 mV vs. Hg/Hg_2SO_4 in 0.5 M H_2SO_4 . In gray, region 1 correspond to the capacitive area while region 2 correspond to the reduction peak. Both are used to determine the real surface area from an electrode.	28
3.4	Ditiotreitol (DTT) structure illustration.	30
3.5	MSMS m/z 177 is the ion that was selected for fragmentation while the m/z is the mass to charge ratio used in the analysis of DTT.	31
3.6	Electrospray ionization (ESI) positive of a ssDNA sample prepared using DTT where the 177 g/mol is present	32
3.7	Electrospray ionization (ESI) negative of a ssDNA sample prepared using DTT where the 177 g/mol is present	33

3.8	Cyclic voltammetry (a) and electrochemical impedance spectroscopy (b) (vs. AgAgCl) for probe immobilization (black) followed by MCH immobilization (red) to block the surface avoiding non specific interaction. Both using 2.5 mM hexacyanoferrate II and III (1:1) in 10 mM Tris, pH 7.4, 100 mV/s	35
3.9	Cyclic voltammetry (a) and electrochemical impedance spectroscopy (b) (vs. Ag/AgCl) for probe immobilization (black) follow by MCH immobilization (red) and hybridization with 1 μ M target DNA. Both using 2.5 mM hexacyanoferrate II and III (1:1) in 10 mM PB, pH 7.4, 100 mV/s	37
3.10	The ssDNA immobilization follow by MCH immobilization is tested over different target DNA concentrations. The CV in (a) shown a shift in the peaks with the target DNA increase while the EIS (b) shown a increase in the R_{ct} in the same conditions.	38
3.11	The R_{ct} values from the Figure 3.10(b) are presented a linear behavior when the concentration of target DNA increase.	39
4.1	The MicroRNA biogenesis process begins in the nucleus where the RNase III enzyme Drosha, coupled with its binding partner DGCR8, cleaves pri-miRNA into pre-miRNA. Pre-miRNAs are exported from the nucleus into the cytoplasm, where the hairpin precursors are cleaved and its binding partner the transactivator RNA-binding protein TRBP into dsRNA duplex that contains both the miRNA strand and its complementary strand. Reproduced from [39].	42
4.2	Chronocoulometric response curves for DNA probe co-immobilized with MCH. In black the SAM in the absence of $Ru(NH_3)_6^{3+}$, in red the SAM in the presence of the complex and in blue, after hybridization with 1 μ M of target DNA and in the presence of $Ru(NH_3)_6^{3+}$	45

4.3	Electrochemical characterization of the SAM using different proportions of probe and spacer. Chronocoulometric response curves in (a) show the SAM in the absence of $\text{Ru}(\text{NH}_3)_6^{3+}$ in black and the different proportions with the presence of the complex. In (b) the EIS response curves in 2.5 mM ferri/ferro in E-BFR at OCP value.	45
4.4	Probe surface density as a function of fraction of DNA to total thiol concentration in immobilization solution. Error bars show the mean and spread for, at least, three samples at each DNA mole fraction. . .	47
4.5	Charge tranfer resistance (R_{ct}) as a function of probe surface density. EIS measurements are preformed in E-BFR with 2.5 mM ferri/ferro at OCP value.	47
4.6	Increase in the charge tranfer resistance (R_{ct}) after hybridization as a function of the fraction of DNA to total thiol. EIS measurements after hybridization are preformed in E-BFR with 2.5 mM ferri/ferro at OCP value.	48
4.7	Data points represent average R_{ct} recorded using EIS from four independent samples at different concentration of tDNA.	48
4.8	The negative control. R_{ct} comparison between non-specific miR-200b and miR-200c sequences hybridization with miR-200a probe in the SAM.	49
4.9	Melting curve of DNA probe and target DNA in E-BFR buffer, pH 7.4. The zero absorbance correspond to the cell with E-BFR. The four different lines with a small increase over the temperature correspond to probes and targets in individual cells. The two curves with a significante increase around 66°C correspond to the cells with probe and target sequence together.	50
4.10	The $\text{HS}-(\text{CH}_2)_6-\text{PO}_4-(\text{CH}_2\text{CH}_2\text{O})_6\text{-ssDNA}$ sequence co-immobilized with MCH in 1:5 molar fraction. In (a) the EIS response to 10nM, 100nM and $1\mu\text{M}$ target DNA. In (b) the R_{ct} increase for each target DNA concnetration.	52

4.11	Real-time curve of frequency response for 3th overtone upon small target DNA concentration in flow-condition. Arrows indicate the injection of target DNA over the time.	54
4.12	Real-time curve of frequency response for 3th overtone upon increasing target DNA concentration in flow-condition. Arrows indicate the injection of target DNA and E-BFR to wash.	54
4.13	Information from Table 4.2. The frequency response over the target DNA concentration in (a) and the change of mass over the target DNA concentration in (b).	55
4.14	Difference of potential as a function of the target DNA concentration. Hybridization is performed at E-BFR without redox marker in solution.	56
5.1	Predicted aptamer 1 secondary structures. Reproduced from [51]. . .	60
5.2	Influence of different molar fraction in R_{ct} . Electrodes were co-immobilized with HER2 aptamer and MCH in different molar fraction. The bars show the R_{ct} after $1\mu\text{M}$ HER2 binding for 30min.	61
5.3	SPR specific and non-specific binding. The aptamer was tested with 50nM of HER2 and AT-PSA.	62
5.4	Predicted aptamer secondary structures for probe 2. Reproduced from [52].	62
5.5	Aptasensor detection. Co-immobilized aptamer with MCH was tested with 1:5, 1:10, 1:50 and 1:100 molar fractions with $1\mu\text{M}$ of HER2 protein (a). The optimized molar fraction (1:50) was tested with different HER2 concentrations using EIS (b).	63
A.1	Horizontal gel electrophoresis apparatus with the sample inside. Reproduced from [61]	76
A.2	Example of a thermal profile. The amplification curve will be performed on the segment 3. Reproduced from [63]	79
A.3	Example of amplification curve.	79
A.4	Workflow for qPCR	85
A.5	Assembly of a sandwich in Western Blot. Reproduced from [65]. . . .	90
A.6	Cell culture flasks before incubation.	91

A.7	MCF-7 cells confluent. Passage number 28.	91
A.8	MCF-7 cells confluent and after 48h in hypoxia. Passage number 28. .	92
A.9	Electrophoresis gel tested with microRNA extracted from MCF7 cells in different passages.	93
A.10	Electrophoresis gel tested with microRNA extracted from MCF7 cells in different passages. The P represents the passage, H hypoxia and C control or normoxia. The experiment was repeated twice.	94
A.11	Amplicification curve for 45 cycles. In blue are the curves from microRNA 200a being tested and in red the housekeeping gene for normalization.	95
A.12	Western Blot result for MCF7 cells exposed to hypoxic conditions. . .	97

LIST OF TABLES

3.1	DNA sequences used in the work. The probe with thiol modification, the full complementary sequence (target), 4 mismatch and a non-complementary sequence. In red is highlighted the mismatch bases.	29
3.2	From the voltammogram in Figure 3.9(a) it is possible to extract the potentials of the anodic and cathodic peak. Also, the difference between them, ΔE , for each step of the immobilization and hybridization.	37
3.3	The main information extracted from EIS (Figure 3.9(b)) is the charge transfer resistance (R_{ct}). This value is shown for each step for, at least, three different electrodes.	37
4.1	ssDNA surface density and charge transfer resistance values are presented for each probe and spacer proportion tested.	46
4.2	Amount of DNA hybridized. From Figure 4.11 and 4.12 were extracted the Δf , Δm and the theoretical amount of DNA.	55
5.1	DNA aptamer sequences. The two sequences tested for aptasensor development.	58
A.1	Reverse transcription reaction setup.	86
A.2	Real - time PCR reaction, pr. $10\mu L$ reaction.	86
A.3	Real-time PCR cycle conditions	86
A.4	Acrylamine gel preparation.	89
A.5	Example of data analyze from P5 cells. The Exp. Ct and Con. Ct values are the average of three results.	96

A.6 $\Delta\Delta Ct$ and $2^{-\Delta\Delta Ct}$ values to different cell passage without use any passive reference dye.	96
A.7 $\Delta\Delta Ct$ and $2^{-\Delta\Delta Ct}$ values to different cell passage using ROX as passive reference dye.	96

ABBREVIATIONS

AC	Alternate current.
BioFET	Biological recognition element immobilized on FET surface.
BVD	Butterworth van Dyke.
CC	Chronocoulometry.
CV	Cyclic voltammetry.
DNA	Deoxyribonucleic acid.
dsDNA	Double strand DNA.
DTT	Dithiothreitol.
EDTA	Ethylenediaminetetraacetic acid.
EIS	Electrochemical impedance spectroscopy.
EGFET	Extended Gate Field-Effect Transistor.
FET	Field-effect transistor.
IHP	Inner Helmholtz phase.
ISFET	Ion Sensitive Field-Effect Transistor.
LNA	Locked nucleic acid.
LOD	Limit of detection.
MCH	6-mercapto-1-hexanol.

miRNA	MicroRNA.
MOSFET	Metal-oxide-semiconductor field-effect transistor.
OHP	Outer Helmholtz phase.
PB	Potassium phosphate buffer.
PBS	Phosphate-buffered saline.
PCR	Polymerase chain reaction.
PNA	Peptide nucleic acid.
QCM	Quartz crystal microbalance.
RC	Resistor Capacitor.
RNA	Ribonucleic acid.
SAM	Self-assembled monolayer.
ssDNA	Single strand DNA.
tDNA	target DNA.
TE	Tris with EDTA.
WB	Western Blot.

NOMENCLATURE

A	Area.
C	Complex capacitance.
C'	Real capacitance.
C''	Imaginary capacitance.
C_0	Concentration of species oxidized.
C_1	Capacitor related to the stored energy in the oscillation.
C_D	Diffuse-layer capacitance.
C_{dl}	Double layer capacitance.
C_H	Helmholts-layer capacitance.
C_Q	Constant of the quartz.
d	Thickness of dielectric layer.
D	Dissipation factor.
D_O	Diffusion coefficients of oxidised form .
D_R	Diffusion coefficients of reducer form.
E	Potential.
E_0	Standard electrode potential or potential peak amplitude.
E_{ap}	Anode peak potential.

E_{cp}	Cathode peak potential.
E_f	Reverse potential.
E_i	Initial potential.
F	Faraday constant.
f	Frequency.
f_q	Quartz resonator frequency.
I or i	Current.
I_0	Peak current amplitude.
I_{ap}	Anode peak current.
I_{cp}	Cathode peak current.
L_1	Inductor related to the inertial component of the oscillation.
n	Number of electrons or overtone.
Q	Total charge.
Q_{ads}	Adsorbed charge.
Q_c	Double layer charge.
Q_{diff}	Cumulative charge.
R	Gas constant or Resistance.
R_1	Dissipation of the oscillation energy.
R_{ct}	Charge transfer resistance.
R_{dl}	Double layer resistance.
R_S	Solution resistance.
T	Temperature.

t	Time.
Y	Admittance.
Z	Complex impedance.
Z' or Z_{Re}	Real impedance.
Z'' or Z_{Im}	Imaginary impedance.
Z_C	Impedance of a capacitor.
Z_R	Impedance of a resistor.
Z_{RC}	Impedance of RC system.
Z_W	Warburg impedance.
Γ_{DNA}	Probe surface density.
Γ_R	Amount of redox marker confined near the electrode surface.
Δ_D	Dissipation factor shift.
ΔE_p	Distance separation of peak potential.
Δ_f	Frequency shift.
Δ_m	Change of mass.
ε_0	Permittivity of free space.
ε_m	Dielectric constant of SAM.
η_l	Liquid density.
θ	Phase angle from potential.
μ_q	Shear modulus.
ρ_l	Liquid viscosity.
ρ_q	Quartz density.

ϕ	Phase angle from current.
ω	Angular frequency.

CONTENTS

List of Figures	ix
List of Tables	xv
Abbreviations	xvii
Nomenclature	xix
1 Introduction	1
1.1 Introduction	1
1.2 Aim of the study	2
1.3 Overview of the thesis	3
2 Theories and Principles	5
2.1 Electrochemical overview of the system	5
2.2 Principles of Electrochemistry	6
2.2.1 Electrical Double Layer	6
2.2.2 Electrochemical Cells	7
2.2.3 Electrochemical characterization techniques	9
2.2.3.1 Cyclic Voltammetry	9
2.2.3.2 Chronocoulometry	11
2.2.4 Impedance techniques	13
2.2.5 Electrochemical Impedance Spectroscopy	13
2.2.6 Electrochemical Capacitance Spectroscopy	18
2.3 Principles of Piezoelectricity	19
2.3.1 Quartz Crystal Microbalance	20

2.4	Principles of Field-Effect Transistors	21
2.4.1	Extended Gate Field-Effect Transistor	22
3	DNA Probe immobilization	24
3.1	DNA self-assembled monolayer	24
3.2	Pretreatment of gold electrodes	26
3.2.1	Cleaning procedure	26
3.2.2	Determination of the surface roughness	27
3.3	Sample preparation	29
3.3.1	Complementary sequences	29
3.3.2	Thiol-modified probe	29
3.4	DNA probe immobilization strategies	34
3.4.1	Weak ionic strength immobilization buffer	34
3.4.2	Strong ionic strength immobilization buffer	35
3.4.3	Co-immobilization with a strong ionic strength buffer	38
4	DNA hybridization detection	40
4.1	MicroRNA	41
4.2	Biosensor Fabrication	42
4.2.1	Materials	42
4.2.2	Sample preparation	43
4.2.3	Electrode preparation	43
4.2.4	Instrumentation	43
4.3	Electrochemical characterization of modified electrodes	44
4.3.1	DNA surface density	44
4.4	Hybridization Troubleshooting	49
4.4.1	Thermal Melt Analysis	49
4.4.2	Linker	51
4.5	BioFET and QCM sensor for DNA hybridization	52
4.5.1	Hybridization detection using QCM	53
4.5.2	BioFET detection	56

5	HER2 aptamer	57
5.1	Biosensor Fabrication	58
5.1.1	Materials	58
5.1.2	Sample preparation	58
5.1.3	Electrode preparation	59
5.1.4	Instrumentation	59
5.2	Electrochemical characterization of modified electrodes	60
6	Conclusion	64
	References	67
A	Biological Aspects	74

INTRODUCTION

1.1 Introduction

IN April 1953 James Watson and Francis Crick proposed in a simple article the correct double helix model for DNA structure through x-ray diffraction [1]. This discovery has paved the way for all understanding of how genetic information is stored and transmitted. Almost 10 years later, Eley and Spivey suggested that DNA could conduct charge based on its similarity to one-dimensional structures of aromatic crystals [2]. Since then, the interest in the "molecule of life" has increased.

The oligonucleotides detection have been explored in many research and technological fields from forensics to medical diagnostics. There is a significant need for devices with high sensitive and selective detection, and also low cost, giving a direct electronic signal and that can easily be miniaturized to portable devices for point-of-care [3]. For this, biosensors have been investigated once they can provide specific quantitative information using a biological recognition element directly in contact with a transduction element. Specifically, a DNA biosensor presents a single strand nucleic acid working as biological recognition element [4]. Different detection strategies have been applied, however there are just a few commercial devices. Problems with reproducibility and sensitivity are the major concern due to surface attachment and hybridization.

The electrochemical transducer attracted a lot of attention since it gives an electronic signal directly, with low cost and can easily be miniaturized, enabling the development of portable devices [5]. Also, several approaches to signal transduction presented promising results [6]. The development of this biosensors with broad

potential for exploitation and, therefore, eliminate problems of reproducibility and sensitivity.

Therefore, electrochemical biosensors are one of the most promising platforms with the potential to achieve these goals and have already been used to detect different analytes, including, e.g., disease biomarkers [7] and proteins [8], microRNAs [6], microorganisms in water [9], toxins in food samples [10] and biomolecules in human serum [11].

1.2 Aim of the study

The aim of the thesis is to develop a DNA/DNA biosensor that can be easily expanded to microRNA, PNA or aptamer systems. The characterization and detection are based mainly on DNA charge using electrochemical techniques, but also will be compared with piezoelectric and field-effect techniques. The development involves a deep understanding of DNA immobilization, electrochemical double-layer components and how the biorecognition event affects the double-layer.

The electrochemical biosensor platform focuses on synthetic DNA oligonucleotides immobilized via thiol groups onto gold electrodes surface, where the single strand DNA has intrinsic negative charge due to its phosphate groups. Hybridization of the complementary sequence increase the negative charge in the electrode surface and the detection of this label-free system through electrochemical impedance spectroscopy requires a redox marker in solution. However, the same system can be further amplified with gold nanoparticles or redox intercalators and modulated by other electrochemical techniques, as square wave voltammetry or electrochemical capacitance spectroscopy.

The biosensor platform was tested with piezoelectric and field-effect transduction. In the first case, the immobilization strategy was reproduced on the top of a quartz crystal covered with gold. The hybridization increases the mass, decreasing the resonance frequency. On the other hand, field-effect transducers are able to detect directly the charge variation. For this, gold electrodes were connected to the gate of the FET. The limits of detection for all techniques are compared.

To show that the DNA/DNA biosensor can be expanded to other systems

with a similar immobilization strategy, an aptasensor is developed using different DNA sequences to detect a target protein. Surface plasmon resonance confirms the protein binding. All the electrochemical biosensors were modeled by an equivalent electric circuit.

1.3 Overview of the thesis

Chapter 2 introduces the basic theories and relevant aspects for the DNA biosensors employed in the study. It starts with an overview of the system, reviewing the electrochemical double layer, the electrochemical cell and the main techniques involved. It is followed by the theories that describe piezoelectric and field-effect transducers.

The study of probe immobilization is presented in chapter 3, which includes: gold electrodes cleaning, determination of surface roughness, sample preparation and the self assembled monolayers (SAM). All these steps proved to be necessary in order to develop a compact and stable monolayer and reproducible results. Immobilization procedure was optimized and will be discussed in detail.

In chapter 4 electrochemical impedance spectroscopy (EIS) of modified electrode surfaces is studied based on the charge effect. To achieve better sensitivity and lower detection limit, the SAM was optimized using electrochemical methods and modeled according to an electric circuit to understand the significance of all elements in the mixed structure and solutions. Also included a brief introduction on the microRNA sequence used and its role as a biomarker. A brief comparative study of DNA hybridization sensors using quartz crystal microbalance and field effect transistor signal is presented.

Chapter 5 introduces the aptasensors using different DNA sequences for HER2 protein and its importance as a biomarker. The same immobilization strategy on the top of gold electrodes was investigated using EIS. The density of aptamers on the surface was optimized to achieve limits of detection close to the clinically relevant values.

The final conclusions are presented in chapter 6 with suggestions on how this work can be improved and extended to other biosensors systems.

At the same time, it is important to understand biological aspects of targets molecules. The appendix in the end of the thesis presents a cancer cell line culture and how to extract specific microRNAs. Different biological techniques can be used to describe and evaluate this material. For microRNA quantification, polymerase chain reaction (PCR) amplification is the main technique. Some important aspects will be discussed to correlate human levels with the biosensors limits of detection.

CONCLUSION

Label-free DNA biosensor was investigated to detect target analyte in solution using different techniques. Electrochemical transduction was explored through cyclic voltammetry, electrochemical impedance spectroscopy and chronocoulometry, followed by quartz crystal microbalance and biofet where the same parameters were reproduced. The oligo sequence used as probe and target correspond to a DNA form of microRNA 200a, related with breast and ovary cancer.

The platform was first optimized starting with the immobilization procedure, including electrode cleaning, probe and spacer immobilization, buffers and probe density quantification. The electrode surface roughness or the real area was determined by electrochemical cleaning and limited to 1.5 x geometric area. High ionic strength immobilization buffer combined with co-immobilization of probe and spacer gives a better quality in terms of probe orientation, control of probe density and reproducibility. This procedure followed by backfilling with high concentration of spacer avoid non-specific interaction.

Immobilization procedure was optimized using cyclic voltammetry and electrochemical impedance spectroscopy. The probe surface density was determined through chronocoulometry. For that, a mix using different molar fraction of probe and spacer were prepared in the immobilization buffer. This method provide an effective control of probe surface density. A linear relationship between molar fraction and probe surface density was observed changing the probe from 10 to 50% and getting as result from 2.84 to 4.31 x 10¹² molecules/cm².

The optimized system was tested with 1 μ M target sequence and the increase of R_{ct} was compared to probe density to obtain the optimized hybridization

efficiency. In this case, 20% of probes on the immobilization buffer leads to the highest increase of the R_{ct} due to hybridization. From these parameters was possible to set up a calibration curve, where 100 fM as limit of detection was observed. This limit is high compared with the literature, however any amplification was used and the platform was able to distinguish non-specific sequences with 4 mismatch bases decreasing more than 80% of the R_{ct} .

The platform was reproduced on top of QCM electrodes considering the same parameters. The frequency decrease with the hybridization over different target concentrations. The frequency can be used to estimate the density of target sequences that hybridized. However, the values are higher than the probe surface density estimated electrochemically. In this case, the real area of QCM electrodes are higher due to its roughness, which also affects the area value used on Cottrell equation.

The bulk gold electrode modified with SAM was also connected with the gate of the EGFET system using an instrumental amplifier. The charge increased on the electrode surface due to hybridization was just able to detect high concentrations of target DNA. This low cost detection has to be improved with further signal amplification.

For the system used in this work, further improvement on performance are required, such as nanoparticles or DNA intercalators for signal amplification. The electrode modification presented here has a classical approach, however, different surface modifications already reported show it as a possibility.

Aptamers are single strand of DNA or RNA that are designed to bind proteins with high affinity and specificity. The same SAM was developed replacing the short ssDNA by DNA aptamer with a longer sequence. In this case, two different HER2 aptamers bind the target with different dissociation coefficient and have different sequence length. The effect of these parameters are reflected on the binding event identification. The increase of sequence length increases the binding event from the SAM surface, decreasing the detection. At the same time, low dissociation coefficient are required to increase the detection. Playing with these parameters were possible to optimize the platform for HER2 detection. However, further improvements are required to distinguish low variations on clinically relevant values.

Promising results have been achieved from the study of DNA hybridization sensor system. The electrochemical impedance spectroscopy proved to be a versatile technique for biomarker detection supported by other electrochemical techniques. The biosensor platform can also be reproduced using other transducing or even others biomarkers. This first study opened the way for a lot of possibilities of biomarker detection and improvements.

REFERENCES¹

- [1] WATSON, J. D.; CRICK, F. H. C. Molecular structure of nucleic acids. *Nature*, p. 737–738, 1953.
- [2] ELEY, D.; SPIVEY., D. I. Semiconductivity of organic substances. part 9. nucleic acid in the dry state. *Trans. Faraday Soc.*, v. 58, p. 411, 1962.
- [3] DRUMMOND, T. G.; HILL, M. G.; BARTON, J. K. Electrochemical dna sensors. *Nature Biotechnology*, v. 21, p. 1192 – 1199, 2003.
- [4] THEVENOT, D. R. et al. Electrochemical biosensors: recommended definitions and classification. *Biosensors & Bioelectronics*, v. 16, p. 121–131, 2001.
- [5] KEIGHLEY, S. D. et al. Optimization of dna immobilization on gold electrodes for label-free detection by electrochemical impedance spectroscopy. *Biosensors and Bioelectronics*, v. 23, p. 1291–1297, 2008.
- [6] JOLLY, P. et al. Highly sensitive dual mode electrochemical platform for microrna detection. *Scientific Reports*, v. 6, p. 36719, 2016.
- [7] GOPINATH, S. C. et al. Aptamer-based 'point-of-care testing. *Biotechnology Advances*, v. 34, p. 198–208, 2016.
- [8] FORMISANO, N. et al. Optimisation of an electrochemical impedance spectroscopyaptasensor by exploiting quartz crystal microbalance with dissipation signals. *Sensors and Actuators B*, v. 220, p. 369–375, 2015.
- [9] DENNISON, M. J.; TURNER, A. P. F. Biosensors for environmental monitoring. *Biotechnology Advances*, p. 1–12, 1995.

¹According to Brazilian Association of Technical Standards (ABNT). NBR 6023.

- [10] PRODROMIDIS, M. I.; KARAYANNIS., M. I. Enzyme based amperometric biosensors for food analysis. *Electroanalysis*, p. 241–261, 2002.
- [11] TSIAFOULIS, C. G.; PRODROMIDIS, M. I.; KARAYANNIS, M. I. Development of amperometric biosensors for the determination of glycolic acid in real samples. *Analytical Chemistry*, p. 132–139, 2002.
- [12] BARD, A. J.; FAULKNER, L. R. *Electrochemical Methods: Fundamentals and Applications*. 2nd. ed. [S.l.]: Wiley, 2000. ISBN 978-0-471-04372-0.
- [13] WANG, H.; PILON, L. Accurate simulations of electric double layer capacitance of ultramicroelectrodes. *J. Phys. Chem. C*, v. 115, p. 16711–16719, 2011.
- [14] STOJEK, Z. *Electroanalytical Methods*. 2nd. ed. [S.l.]: Springer, 2009. ISBN 978-3-642-02914-1.
- [15] BRADSHAW, L. Understanding piezoelectric quartz crystals. *RF time and frequency*, v. 8, p. 50–58, 2000.
- [16] RODAHL, M. et al. Simultaneous frequency and dissipation factor qcm measurements of biomolecular adsorption and cell adhesion. *Faraday Discuss*, v. 107, p. 229–246, 1997.
- [17] SAUERBREY, G. Verwendung von schwingquarzen zur wagung dunner schichten und zur mikrowagung. *Zeitschrift fur Physik*, v. 155, p. 206–222, 1959.
- [18] NOMURA, T.; MINEMURA, A. Behavior of a piezoelectric quartz crystal in an aqueous solution and the application to the determination of minute amount of cyanide. *Chemical Society of Japan*, v. 10, p. 1621–1625, 1980.
- [19] BARD, A. J.; FAULKNER, L. R. *Electrochemical DNA Biosensors*. 1st. ed. [S.l.]: Pan Stanford Publishing Pte. Ltd., 2012. ISBN 978-981-4303-98-9.
- [20] BERGVELD, P. Development of an ion-sensitive solid-state device for neurophysiological measurements. *Ieee Trans, Biomed. Eng.*, v. 70, p. BM17, 1970.
- [21] SPIEGEL, J. van der et al. Teh extended gate chemically sensitive field effect transistor as multi-species microprobe. *Sens. Actuators*, v. 4, p. 291–298, 1983.

- [22] LOVE, J. C. et al. Self-assembled monolayers of thiolates on metals as a form of nanotechnology. *Chemical Reviews*, v. 4, p. 1103–1169, 2005.
- [23] CARVALHAL, R. F.; FREIRE, R. S.; KUBOTA, L. T. Polycrystalline gold electrodes: A comparative study of pretreatment procedures used for cleaning and thiol self-assembly monolayer formation. *Electroanalysis*, v. 17, p. 1251–1259, 2005.
- [24] TOMBELLI, S.; MASCINI, M.; TURNER, A. P. F. Improved procedures for immobilisation of oligonucleotides on gold-coated piezoelectric quartz crystals. *Biosensors & Bioelectronics*, v. 17, p. 929–936, 2002.
- [25] STEEL, A. B.; HERNE, T. M.; TARLOV, M. J. Electrostatic interactions of redox cations with surface-immobilized and solution dna. *Bioconjugate Chemistry*, v. 10, p. 419–423, 1999.
- [26] STEEL, A. B. et al. Immobilization of nucleic acids at solid surfaces: Effect of oligonucleotide length on layer assembly. *Biophysical Journal*, v. 79, p. 975–981, 2000.
- [27] HOOGLIET, J. C. et al. Electrochemical pre-treatment of polycrystalline gold electrodes to produce a reproducible surface roughness for self assembly: A study in phosphate buffer ph 7.4. *Analytical Chemistry*, v. 72, p. 2016–2021, 2000.
- [28] RON, H.; MATLIS, S.; RUBINSTEIN, I. Self-assembled monolayers on oxidized metals. 2. gold surface oxidative pretreatment, monolayer properties, and depression formation. *Langmuir*, v. 15, p. 1116–1121, 1998.
- [29] RON, H. H.; RUBINSTEIN, I. Alkanethiol monolayers on preoxidized gold-encapsulation of gold oxide under an organic monolayer. *Langmuir*, v. 10, p. 4566–4573, 1994.
- [30] LAO, R. J. et al. Electrochemical interrogation of dna monolayers on gold surfaces. *Analytical Chemistry*, v. 77, p. 6475–6480, 2005.

- [31] KHOSRAVINIA, H.; RAMESHA, K. P. Influenca of edta and magnesium on dna extraction from blood samples and specificity of polymerase chain reaction. *African Journal of Biotechnology*, v. 6, p. 184–187, 2007.
- [32] STEEL, A. B.; HERNE, T. M.; TARLOV, M. J. Electrochemical quantitation of dna immobilized on gold. *Analytical Chemistry*, v. 70, p. 4670–4677, 1998.
- [33] ESTRELA, P. et al. Label-free sub-picomolar protein detection with field-effect transistors. *Anal. Chem.*, v. 82, p. 3531–3536, 2010.
- [34] LUCARELLI, F. et al. Electrochemical and piezoelectric dna biosensors for hybridization detection. *Anal. Chem.*, v. 602, p. 139–159, 2008.
- [35] HU, P. et al. Self-assembled nanotube field-effect transistors for label-free protein biosensors. *Journal of Applied Physics*, v. 104, p. 074310, 2008.
- [36] HENEGHAN, H. M.; MILLER, N.; KERIN, M. J. Mirnas as biomarkers and therapeutic targets in cancer. *Current Opinion in Pharmacology*, v. 10, p. 543–550, 2010.
- [37] LEE, R.; FEINBAUM, R. L.; AMBROS, V. The c. elegans heterochronic gene lin-4 encodes small rnas with antisense complementarity to lin-14. *Cell*, v. 75, p. 843–54, 1993.
- [38] ZHANG, B. et al. micrnas as oncogenes and tumor suppressors. *Developmental Biology*, v. 302, p. 1–12, 2007.
- [39] ESTRELA, P. *Essays in Biochemistry*. 1st. ed. [S.l.]: Portland Press, 2016. ISBN 978-1-85578-199-3.
- [40] HUMPHRIES, B.; YANG, C. The microrna-200 family: small molecules with novel roles in cancer development, progression and therapy. *Oncotarget*, v. 6, p. 6472–98, 2015.
- [41] SIEGEL, R.; NAISHADHAM D., J. A. D. Cancer statistics,2013. *CA Cancer J. Clin.*, v. 63, p. 11–30, 2013.

- [42] DIACONU, I. et al. Electrochemical immunosensors in breast and ovarian cancer. *Clin. Chim. Acta*, v. 425, p. 128–138, 2013.
- [43] PATRIS, S. et al. Nanoimmunoassay onto a screen printed electrode for her2 breast cancer biomarker determination. *Talanta*, v. 130, p. 164–170, 2014.
- [44] HUNG, M. et al. Her-2 neu-targeting gene therapy - a review. *Gene*, v. 159, p. 65–71, 1995.
- [45] CHUN, L. et al. Electrochemical detection of her2 using single stranded dna aptamer modified goldnanoparticles electrode. *Sens. Actuat. B: Chem.*, v. 186, p. 446–450, 2013.
- [46] PRESS, M. et al. Evaluation of her-2/neu gene amplification and overexpression: comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimens. *J. Clin. Oncol.*, v. 20, p. 3095–3105, 2002.
- [47] CAMACHO, C. et al. Novel enzyme biosensor for hydrogen peroxide via supramolecular associations. *Biosens. Bioelectron.*, v. 24, p. 2028–2033, 2009.
- [48] NOLTE, D. Prostate-specific antigen immunoassays on the biocd. *Anal. Bioanal. Chem.*, v. 393, p. 1151–1156, 2009.
- [49] QURESHIA, A.; GURBUZB, Y.; NIAZIA, J. Label-free capacitance based aptasensor platform for the detection of her2/erbb2 cancer biomarker in serum. *Sensors and Actuators B*, v. 220, p. 1145–1151, 2015.
- [50] CHO, E.; LEE, J.; ELLINGTON, A. Applications of aptamers as sensors. *Ann. Rev. Anal. Chem.*, v. 2, p. 241–264, 2009.
- [51] LIU, Z. et al. Novel her2 aptamer selectively delivers cytotoxic drug to her2-positive breast cancer cells in vitro. *Journal of Translational Medicine*, v. 10, p. 148, 2012.
- [52] GIJS, M. et al. Improved aptamers for the diagnosis and potential treatment of her2-positive cancer. *Pharmaceuticals*, v. 9, p. 29, 2016.

- [53] HOLLIDAY, D.; SPEIRS, V. Choosing the right cell line for breast cancer research. *Holliday and Speirs Breast Cancer Research*, v. 13, p. 215, 2011.
- [54] ALTOGEN. *MCF-7 Cells human breast adenocarcinoma cell line*. 2016. <http://www.mcf7.com>. [Online; accessed 15-Jun-2016].
- [55] BARTEL, D. P. Micrnas: genomics, biogenesis, mechanism, and function. *Cell*, v. 116, p. 281–97, 2004.
- [56] LAWRIE, S. G. C. H.; DUNLOP, H. M. Detection of elevated levels of tumour-associated micrnas in serum of patients with diffuse large b-cell lymphoma. *Br J Haematol*, v. 141, p. 672–5, 2008.
- [57] CUK, K. et al. Circulating micrnas in plasma as early detection markers for breast cancer. *Int. J. Cancer*, v. 132, p. 1602–1612, 2013.
- [58] JURMEISTER, S. et al. Microrna-200c represses migration and invasion of breast cancer cells by targeting actin-regulatory proteins fhod1 and ppm1f. *Molecular and Cellular Biology*, v. 32, p. 633–651, 2012.
- [59] BANDO MASAKAZU TOI, K. K. H.; KOIKE, M. Genes commonly upregulated by hypoxia in human breast cancer cells mcf-7 and mda-mb-231. *Biomedicine & Pharmacotherapy*, v. 57, p. 333–340, 2013.
- [60] HAMDAN, F. H.; ZIHLIF, M. A. Gene expression alterations in chronic hypoxic mcf7 breast cancer cell line. *Genomics*, v. 104, p. 477–481, 2004.
- [61] WESTERMEIER, R. Gel electrophoresis. *ENCYCLOPEDIA OF LIFE SCIENCES*, p. 1–6, 2005.
- [62] COMPANY, T. B. E. *Principles and Practice of Agarose Gel Electrophoresis*. 2016. <https://www.wou.edu/las/physci/ch462/Gel%20Electrophoresis.pdf>. [Online; accessed 15-Jun-2016].
- [63] EUROGENTEC. *qPCR Guide*. 2016. <http://www.eurogentec.com/uploads/qPCR-guide.pdf>. [Online; accessed 15-Jun-2016].

-
- [64] RAD, B. *What is Real-Time PCR (qPCR)?* 2016. <http://www.bio-rad.com/pt-br/applications-technologies/what-real-time-pcr-qpcr>. [Online; accessed 15-Jun-2016].
- [65] MAHMOOD, T.; YANG, P.-C. Western blot: Technique, theory, and trouble shooting. *North American Journal of Medical Sciences*, v. 4, p. 429–34, 2012.
- [66] SHEN, G. et al. Hypoxia-regulated micornas in human cancer. *Acta Pharmacol Sin*, v. 34, p. 336–341, 2013.