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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.

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MARINA RIBEIRO BATISTUTI

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Prof. Dr. : _____ Institution: _____

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Prof. Dr. : _____ Institution: _____

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Prof. Dr. : _____ Institution: _____

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Prof. Dr. : _____ Institution: _____

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Prof. Dr. : _____ Institution: _____

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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

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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 x 10^{12} molecules/cm^2. 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 resistence (20%) upon 1 μM 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 amplifing 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.

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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 phophate buffer.
PBS	Phosphate-buffered saline.
PCR	Polymerase chain reaction.
PNA	Peptide nucleic acid.
QCM	Quartz cristal 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	Admitance.
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	Dieletric 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

Chapter 1

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 eletronic 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 tranducer attracted a lot of attention since it gives an eletronic 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 piezoeletric 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 piezoeletric and field-effect transduction. In the first case, the immobilization strategy was reproduced on the top of a quartz cristal covered with gold. The hybridization increases the mass, decreasing the resonance frequence. On the other hand, field-effect tranducers 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 eletric 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 piezoeletric 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 eletric 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 crytal 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 relevante 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.

Chapter 6

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 cleanning and limited to 1.5 x geometric area. High ionic strenght 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 follow by backfilling with high concentration of spacer avoid non-specific interation.

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 10^{12} molecules/cm^2.

The optimized system was tested with 1 μM 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 hibridization over different target concentrations. The frequency can be used to estimate the density of target sequences that hybridized. However, the values are higher that the probe surface density estimated electrochemically. In this case, the real area of QCM electrodes are higher due to it 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 have 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 different surface modifications already reported show it as a possibility.

Aptamers are single strand of DNA or RNA that as design 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 coeffcient and have different sequence length. The effect of these parameters are reflected on the bind event identification. The increase of sequence length increase the binding event from the SAM surface, decreasing the detection. At the same time, low dissociation coeffcient are required to increase the detection. Playing with these parameters were possible to optimized 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.

References[1]

[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. Farday 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 & Bielectronics*, 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 dissipationsignals. *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.

[1] According 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] HOOGVLIET, 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. microrna 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 terapy. *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 ofher-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. Novelenzyme 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 ofher2/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. Microrna: 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 microrna 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 microrna 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 micrornas in human cancer. *Acta Pharmacol Sin*, v. 34, p. 336–341, 2013.