ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DO PORTO

INSTITUTO POLITÉCNICO DO PORTO

# Development of an electrochemical biosensor for the detection of miRNA-155 in Breast Cancer

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Dissertação submetida à Escola Superior da Tecnologia a Saúde do Porto para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica em Saúde, realizada sob orientação da Doutora Maria Goreti F. Sales, Professora Adjunto, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, do Professor Doutor Rúben Fernandes, Professor Adjunto das Ciências Químicas e das Biomoléculas da Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto, e da Doutora Felismina Moreira, Investigadora, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto.

Dezembro, 2015

*Dedicate this work to my parents, my sister, my fiancé, and grandparents*

*for the encouragement and love in all moments of my life.*

# Acknowledgments

I want to thank my supervisors Prof. Goreti Sales, Prof. Rúben Fernandes and Dra. Felismina Moreira for their support, and encouragement in this work. Their scientific knowledge, teaching expertise, and their enthusiasm for science contributed to both my professional and personal evolution. I thank all of you for the trust in me and my work.

I thank Prof. Goreti Sales for inspiration, patience for hearing me in all moments, for always being available to help me, and for being more than an advisor for me.

I thank Dra. Felismina for helping me every day with her enthusiasm and optimism, never giving up on me, and being my friend.

I thank Prof. Rúben Fernandes for helping me to become a better researcher, and to be available to help me during my work.

To all my colleagues in the BioMark group that, direct or indirectly, contributed to this work. Thank to Ana Moreira, Liliana Truta and Margarida Piloto for staying with at night, Helena Gomes for waking up early and going to the lab, Nádia Ferreira for being my friend, hearing me, and helping when I needed, Alexandra Santos for helping in everything, and other colleagues (Gabriela Martins, Lúcia Brandão, Catarina Vieira, Carolina Hora, Liliana Carneiro, Rui Gomes, Mariana Carneiro, Helena Sá, Manuela Frasco).

To my parents for their patience, presence, affection, and nights awake. Thanks throughout this investment, for letting me always dream, and for supporting me always in the most difficult moments.

To my sister for all the warmth and presence throughout my life.

To Pedro for dedication, affection, unconditional love and support over the years.

My grandparents for supporting and believing always in me.

My best friends for being my pillar, my strength, my joy, and always being by my side.

# Resumo

O cancro da mama é a forma mais frequente de cancro nas mulheres, a nível mundial. A taxa de mortalidade que se lhe associa tem-se mantido mais ou menos constante, isto apesar dos avanços tecnológicos observados recentemente relativamente ao diagnóstico precoce e à terapêutica. Uma das causas deste cenário é a inexistência de biomarcadores séricos validados que permitam a implementação de diagnóstico precoce.

Neste contexto, os miRNAs têm vindo a despertar um especial interesse na comunidade científica, enquanto biomarcadores promissores e emergentes no diagnóstico precoce do cancro. No cancro da mama, os níveis de expressão de diversos miRNAs são significativamente diferentes entre os tecidos normais e os tecidos com a neoplasia, bem como entre subtipos moleculares do cancro da mama diferentes, associados também a prognóstico.

Neste sentido, este trabalho descreve uma revisão sistemática para a identificação do miRNA a ser estudado. Após meta-análise, selecionou-se o miRNA-155 para o desenvolvimento de um biossensor eletroquímico dedicado à deteção deste miRNA em amostras de soro. O biossensor foi construído em três estágios diferentes: (1) imobilização do oligonucleótido complementar tioloado (anti-miRNA-155) na superfície de um elétrodo de ouro impresso (Au-SPE), seguindo-se (2) o bloqueio de ligações não-específicas com ácido mercaptossuccínico e finalizando com a (3) hibridação do respetivo miRNA.

O biossensor detetou concentrações de miRNA da ordem dos $10^{-18}$ mol/L (aM), apresentando uma resposta linear entre 10 aM e 1 nM. O biossensor apresentou ainda um baixo limite de deteção em amostras reais de soro humano, igual a 5.7 aM, e uma boa seletividade quando na presença de outras biomoléculas de grande dimensão, como o antigénio do cancro da mama CA-15.3 e a albumina de soro bovino (BSA).

Esta abordagem simples e sensível demonstrou ser uma estratégia promissora para a análise quantitativa e/ou simultânea de múltiplos miRNA em fluidos fisiológicos, no que concerne à extensão da investigação biomédica na área da monitorização de biomarcadores e ao diagnóstico em *ponto de cuidado*.

**Palavras-Chave:** Cancro da mama; miRNA; miRNA-155; biossensores eletroquímicos; meta-análise.

Breast cancer is one of the most prevalent forms of cancer in women. Despite all recent advances in early diagnosis and therapy, mortality data is not decreasing. This is an outcome of the inexistence of validated serum biomarkers allowing an early prognosis, out coming from the limited understanding of the natural history of the disease.

In this context, miRNAs have been attracting a special interest throughout the scientific community as promising biomarkers in the early diagnosis of cancer. In breast cancer, several miRNAs and their levels of expression are significantly different between normal tissue and tissue with neoplasia, as well as between different molecular subtypes of breast cancer, also associated with prognosis.

Thus, this these presents a meta-analysis that allows identifying a reliable miRNA biomarker for the early detection of breast cancer. In this, miRNA-155 was identified as the best one and an electrochemical biosensor was developed for its detection in serum samples. The biosensor was assembled by following three button-up stages: (1) the complementary miRNA sequence thiol terminated (anti-miRNA-155) was immobilized on a commercial gold screen-printed electrode (Au-SPE), followed by (2) blocking non-specific binding with mercaptosuccinic acid and by (3) miRNA hybridization.

The biosensor was able to detect miRNA concentrations lying in the $10^{-18}$ mol/L (aM) range, displaying a linear response from 10 aM to 1nM. The device showed a limit of detection of 5.7 aM in human serum samples and good selectivity against other biomolecules in serum, such as cancer antigen CA-15.3 and bovine serum albumin (BSA).

Overall, this simple and sensitive strategy is a promising approach for the quantitative and/or simultaneous analysis of multiple miRNA in physiological fluids, aiming at further biomedical research devoted to biomarker monitoring and point-of-care diagnosis.

**Keywords:** Breast cancer; miRNA; miRNA-155; electrochemical biosensors; meta-analysis.

# Index

I. CHAPTER ........ 1
I.1. Introduction ........ 1
I.1.1. Framework ........ 1
I.1.2. List of Publications ........ 2
I.1.2.1. Patent ........ 2
I.1.2.2. Papers published in international scientific journals ........ 2
I.1.2.3. Poster in conference ........ 2
I.1.3. Brief of State-of-art ........ 3
I.1.3.1. Cancer biomarkers ........ 3
I.1.3.2. miRNA-155 as cancer biomarker ........ 3
I.1.3.3. Biosensors ........ 4
I.1.3.3.1. Biorecognition elements ........ 6
I.1.3.3.2. Electrochemical biosensors ........ 7
I.1.3.3.3. Optical biosensors ........ 11
I.1.4. Objectives ........ 13
II. CHAPTER ........ 15
II.1. Experimental & Methods ........ 15
II.1.1. Systematic revision ........ 15
II.1.2. Biosensor assay ........ 15
II.1.2.1. Apparatus ........ 15
II.1.2.2. Electrochemical Procedures ........ 16
III. CHAPTER ........ 17
III.1. Results and Discussion ........ 17
III.1.1. Systematic revision ........ 17
III.1.2. Biosensor assay ........ 17
III.2. Role of miR-145, miR-155 and miR-181a in Breast Cancer Systematic Review ........ 20
III.2.1. Abstract ........ 21
III.2.2. Introduction ........ 22
III.2.3. Material and methods ........ 24
III.2.3.1. Search strategy and study selection ........ 24
III.2.3.2. Data extraction ........ 25
III.2.3.3. On-line databases ........ 25
III.2.4. Results ........ 25
III.2.4.1. miRNA 145 ........ 28
III.2.4.1.1. Role of miRNA 145 in Breast Cancer ........ 28
III.2.4.1.2. Role of miRNA 145 in other signaling pathways ........ 28
III.2.4.2. miRNA 155 ........ 29

III.2.4.2.1. Role of miRNA 155 in Breast Cancer ....29
III.2.4.2.2. Role of miRNA 155 in other signaling pathways ....29
III.2.4.3. MiRNA 181a ....29
III.2.4.3.1. Role of miRNA 181a in Breast Cancer ....29
III.2.4.3.2. Role of miRNA 181a in other signalling pathways ....30
III.2.5. Discussion ....30
III.2.6. Conclusion ....38
III.2.7. References ....39
III.3. A novel and simple approach for monitoring attomolar concentrations of miRNA-155 in Breast Cancer ....43
III.3.1. Abstract ....44
III.3.2. Introduction ....45
III.3.3. Experimental section ....48
III.3.3.1. Apparatus ....48
III.3.3.2. Reagents ....48
III.3.3.3. Solutions ....49
III.3.3.4. Preparation of electrochemical biosensor on Au-SPE ....49
III.3.3.5. UV Characterization ....50
III.3.3.6. AFM analysis ....50
III.3.3.7. Electrochemical Procedures ....50
III.3.3.8. MiRNA-155 assay ....51
III.3.4. Results and Discussion ....51
III.3.4.1. Biosensor assembly ....51
III.3.4.2. Morphological characterization of the biosensor ....53
III.3.4.3. UV Characterization ....54
III.3.4.4. Electrochemical control of the gold surface modifications ....55
III.3.4.5. Analytical Performance of miRNA sensor ....57
III.3.4.6. Regeneration of Au-SPE ....59
III.3.4.7. Selectivity ....61
III.3.4.8. miRNA-155 assay in human serum samples ....62
III.3.5. Conclusions ....64
III.3.6. Acknowledgements ....64
III.3.7. References ....64
IV. CHAPTER ....69
IV.1. Conclusion ....69
V. REFERENCES ....71

# Index of Figures


Figure 1 Overall scheme of biosensor device. ....................5

Figure 2 Biosensing device schematic representation with biorecognition and transduction elements. ..........6

Figure 3 EIS measurements for different cell lines. (A) MCF-7 and (B) B16F10. ....................18

Figure 4 The current model for the biogenesis and post-transcriptional suppression of microRNAs (…)....23

Figure 5 Flow chart of the search of systematic review. ....................25

Figure 6 Schematic illustration of electrochemical biosensor for detection of miRNA-155. (A) Immobilization of Anti-miRNA155 on gold surface; (B) Blocking the non-specific binding; and (C) Hybridization with miRNA-155. ....................52

Figure 7 AFM images in 2D (below) and 3D (on top) views of different materials. (A) Au-SPR (B) Au-SPR/Anti-miRNA155 and (C) Au-SPR/Anti-miRNA155/miRNA-155. ....................54

Figure 8 UV/Vis absorption spectroscopy of the Anti-miRNA155 (A) and .miRNA-155 (B) and respective complex. ....................55

Figure 9 Electrochemical assays for controlling the gold surface modification, in $5.0\times10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M $[Fe(CN)_6]^{4-}$, solution, prepared in in buffer (TRIS with EDTA and NaCl). CV (A), EIS (B) and SWV (C) assays. ....................56

Figure 10 EIS(A) and SWV(C) measurements, and the corresponding calibration curves (B e D), in $5.0\times10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M $[Fe(CN)_6]^{4-}$, in buffer (TRIS with EDTA and NaCl), pH 7.4, with different concentration of miRNA-155. ....................58

Figure 11 Regeneration of the biosensor (in green), at 90ºC (A, B, C) and 60 ºC (D, E, F), controlled by CV (A, D), EIS (B, E) and SWV (C, D), tested in $5.0\times10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M $[Fe(CN)_6]^{4-}$ redox probe. ....................59

Figure 12 Consecutive calibrations in the same chip, first and after regeneration, (A) in EIS and (B) in SWV assays, performed in $5.0\times10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M $[Fe(CN)6]^{4-}$, in buffer (TRIS and EDTA with NaCl), pH 7.4, in the same concentration range of miRNA-155 as before. ....................61

Figure 13 Selectivity behaviour of the biosensor for miRNA-155 (10 pM) against CA-15.3 (30 U/mL) and BSA (0.30 µg/mL), after 30 minutes incubation and for the same redox probed tested before. ....................62

Figure 14 EIS(A) and SWV(C) measurements, and the corresponding calibration curves (B e D), in $5.0\times10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M $[Fe(CN)_6]^{4-}$, in standard solutions prepared in a background of blank human serum, pH 7.4, with different concentration of miRNA-155. ............63

# I. CHAPTER

## I.1. Introduction

### I.1.1. Framework

This thesis is organized in five chapters. **Chapter I** presents a brief state-of-art of current methods and technologies applied to the assessment of miRNAs as cancer biomarkers. A description of recognition elements used for the new design of biosensors is presented next. Several electrochemical and optical systems are also reviewed, emphasizing characteristics that biosensors should exhibit for a successful practical application.

**Chapter II** describes de main aspects of experimental nature, from the selection of miRNA to the development of the biosensor involved in miRNA-155 detection.

**Chapter III** presents the main results of the present research work. It includes the description of the systematic review of miRNA in breast cancer **(chapter III-2).** This part of the study was performed according to systematic reviews guidelines in order to establish the overall diagnostic accuracy of the measurement of microRNA in the diagnosis of breast cancer. The systematic review was performed with 14 studies, involving 1897 patients available for analysis. This systematic review addressed the three types of the most described microRNAs in breast cancer (miRNA-155, miRNA-145, miRNA-181a).

The development of a rapid and sensitive electrochemical biosensor for detection of miRNA-155 was also described in this chapter **(chapter III-3).** For this purpose, the capture probe was immobilized on a gold screen printed electrode (Au-SPE) surface and each step of the modification process was characterized by electrochemical and AFM techniques. The analytical features of the resulting biosensor were studied by different electrochemical techniques. The biosensor was successfully applied to biological fluids showing ultrasensitive limits.

The **Chapter IV** summarizes the main conclusions obtained and presents guidelines for future research work

**Chapter V** lists the references cited herein.

## I.1.2. List of Publications

### *I.1.2.1. Patent*

Cardoso A.R., Moreira F.T.C., Fernandes R., Sales M.G.F., Development of an Electrochemical Biosensor for the detection of the miRNA-155 in Breast Cancer, *Provisional Portuguese Patent request to IPIN*.

### *I.1.2.2. Papers published in international scientific journals*

Cardoso A.R. , Pereira A.C. Moreira F.T.C., Sales M.G.F., Prudêncio C., Fernandes, R., Role of miR-145, miR-155 and miR-181a in Breast Cancer – Systematic Review (*Submitted for publication*).

Cardoso A.R., Moreira F.T.C., Fernandes R., Sales M.G.F., Development of an Electrochemical Biosensor for the detection of the miRNA-155 in Breast Cancer (*Submitted for publication*).

### *I.1.2.3. Poster in conference*

Cardoso A.R., Fernandes R., Moreira F.T.C., Sales M.G.F., Development of a biosensor for miRNA in Breast Cancer. Poster, J9, presented in the 1$^{st}$ ASPIC *International Conference, the Portuguese Association of Cancer Research, Portugal, Fundação Calouste Gulbenkian*, Lisboa, Portugal (2014).

## I.1.3. Brief of State-of-art

### *I.1.3.1. Cancer biomarkers*

Breast cancer is considered the leading cause of death of women in the world. Actually, several diagnostic and therapeutic innovations have been reported, but their effect on mortality is still discrete. It can be due the little understanding about the natural history of the disease, concerning mainly the progression from *in situ* to invasive breast carcinoma, which is a life-threatening condition. In addition, there are no validated serum biomarkers for early prognosis that are sensitive and specific enough to be used routinely.

In this context, miRNA have been gaining interest within the scientific community as biomarker of early prognosis in cancer disease (Perry et al., 2008). The miRNAs are non-coding RNA species, containing 18-24 nucleotides. They regulate gene expression post-transcriptionally via specific interaction with the 3 'UTR sequence of the target mRNA, resulting in the translation inhibition and/or mRNA degradation. They also play an essential role in biological processes such as development, cell proliferation, apoptosis, stress response, and tumorigenesis (Fu, Chen, & Man, 2011).

Their discovery and functional understanding have only been disclosed in the past ten years. Long large primary transcripts (pre-miRNAs) are transcribed from the genome and processed into pre-miRNAs by Dicer; and then into short single stranded mature miRNAs complexed with Argonaute proteins to inhibit protein translation. The first link of miRNAs to cancer was made only recently, in 2007, but the field has expanded exponentially since (Campuzano, Pedrero, & Pingarrón, 2014).

The expression levels of various miRNAs in breast cancer differ significantly from healthy states. Differences are observed between normal and neoplasic tissues, and within different molecular subtypes of breast cancer diseases that show unrelated prognosis and different responses to therapy. In addition, aberrant expression miRNAs have been observed in several breast cancer solid tumours (Riaz et al., 2013).

### *I.1.3.2. miRNA-155 as cancer biomarker*

In order to understand which miRNA could exhibit suitable biomarker properties in breast cancer, a systematic review of miRNA was described (Chapter III-2) in this thesis. This review allowed confirming that miRNA-155 showed relevant scientific evidence as biomarker in breast cancer.

The miRNA-155 has been previously shown up-regulated in breast cancer, thus being considered as an *oncoMIR*. Deregulation of its expression is involved in numerous pathological processes and oncogenic signalling. In addition, miRNA-155 was significantly increased in sera from breast cancer patients compared with normal controls (Mattiske, Suetani, Neilsen, & Callen, 2012). Furthermore, miRNA-155 expression was inversely correlated with estrogen receptor (ER) and progesterone receptor (PR) expression, regardless of epidermal growth factor receptor-2 (HER-2) status. These findings suggest that miRNA-155 expression is deregulated in breast cancer and is involved in the oncogenesis of breast tumours (Zeng, Fang, Nam, Cai, & Long, 2014). Besides, the overexpression of miRNA-155 is considered as a breast cancer risk factor and some studies showed it can be associated to clinical-pathological markers, tumour subtype, poor survival rates, metastasis events and invasive properties of breast cancer, as well as high tumour grade, and advanced stage lymph node metastasis (Lautner & Gyurcsányi, J. Liu, Huang, Yang, & Luo, 2015).

The existence of a potential biomarker, or set of miRNA biomarkers, miRNA-155 in this case, opens doors towards to a simple serological test for the prognosis/diagnosis of breast cancer or monitoring the disease progression against therapy in point-of-care (POC). This creates the clinical need for a device capable of performing this analysis *on-site* (Zeng et al., 2014).

Thus, a suitable method for miRNA-155 detection in biological fluids is strongly and urgently required. Amongst the wide range of standard techniques that may be employed for the determination of miRNA-155 in serum samples, requirements for sensitivity, rapidity, robustness, reliability and low-cost are fundamental, with biosensors meeting most of these features. Biosensors are currently an alternative to conventional methods like Northern blotting and *in situ* Hybridization, providing fast results, being portable and allowing direct sample reading, thereby avoiding the need to move samples to the laboratory (Hamidi-Asl, Hasheminejad, & Mascini, 2013).

#### *I.1.3.3. Biosensors*

Biosensors are devices converting the interaction of a biorecognition element with a target analyte into a measurable signal, mostly of optical or electrical nature. These devices combine the specificity of interaction of an analyte of interest with a biological/chemical component, called (bio)recognition element, with the sensitivity of a transducer that converts such interaction into a measurable signal that is proportional to the analyte

concentration (Figure 1). The specificity/sensitivity of a biosensor are therefore depending directly of the specificity/sensitivity of the (bio)recognition element for the target compound (Grieshaber, MacKenzie, Vörös, & Reimhult, 2008).

The construction of any biosensor depends primarily of the targeted application. Several relevant features are included herein, such as sensitivity requirements, sample characteristics (composition, stability, volume available), cost and lifetime. Furthermore, the application of a biosensor as an analytical tool in real conditions is restricted to specific features, namely, high selectivity, wide range of concentrations, accuracy and precision, short response time and recovery, high sampling frequency and operating stability, and high reproducibility (Marli & Paiva, 2011).

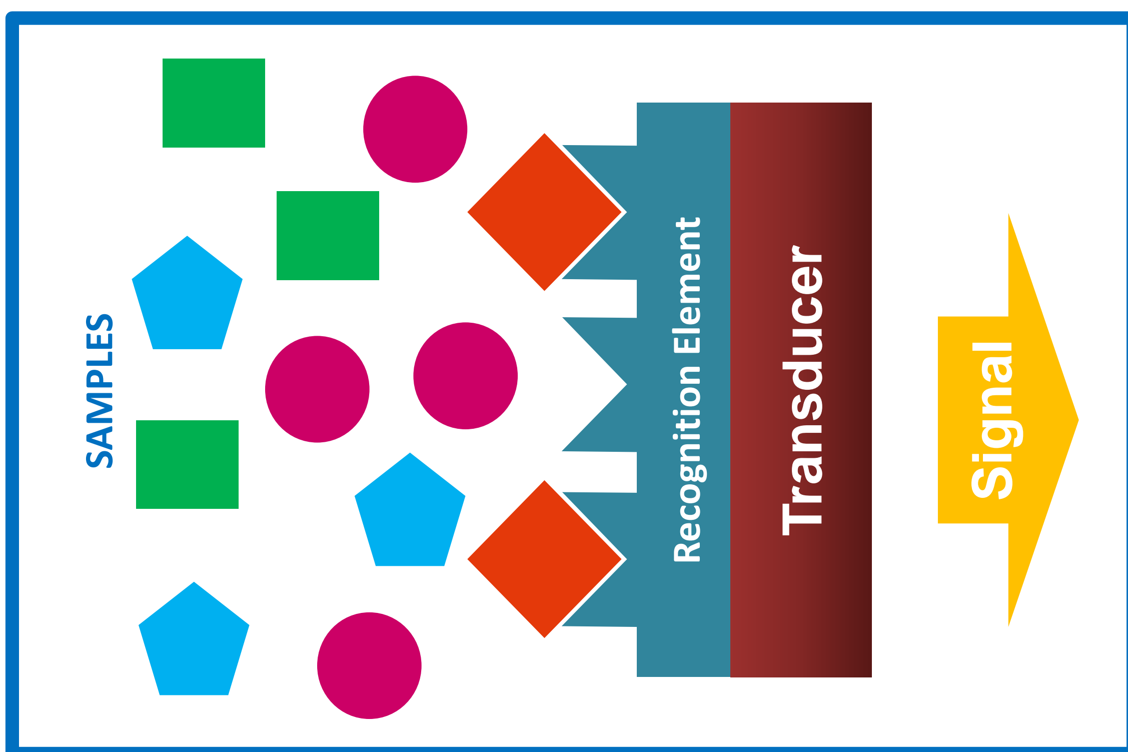


**Figure 1.** Overall scheme of biosensor device.

The (bio)recognition element is a crucial part in a biosensor, since it determines the degree of selectivity of the device. Typically, biologically-based recognition elements used, such as biomolecules, organelles or cells. But synthetic recognition elements, such as biomimetic materials that are prepared in the laboratory and designed to mimic a specific biological property (Figure 2), may also be employed, offering greater stability and reproducibility than their natural counterparts (Bohunicky & Mousa, 2011).

The range of analyte concentrations to which the device is sensitive is often associated with the selected transduction system. In this context, both electrochemical and optical devices are popular, yielding mostly fast measures, of low cost, and automation feasibility. Thus, the selection of a suitable combination of recognition element and transducer is essential when developing a biosensor (Su, Jia, Hou, & Lei, 2011).

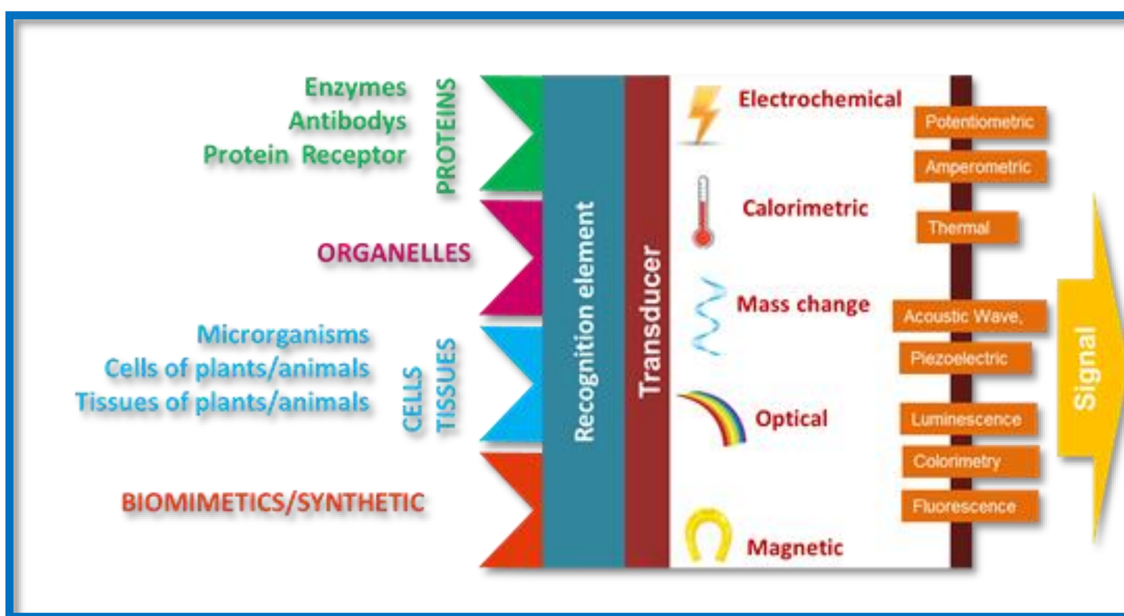


**Figure 2.** Biosensing device schematic representation with biorecognition and transduction elements.

### *I.1.3.3.1. Biorecognition elements*

Each recognition element must contain a binding site for interacting with a target analyte present. This interaction should be stable against environment and measurement conditions and its mode of actuation varies mostly with the chemical nature of this element and its analyte. It can range from affinity processes, such as those related to the interaction between an antibody and its antigen, to catalytic reactions, resulting from the action of an enzyme on its substrate. Indeed, enzymes and antibodies are the most used recognition elements in biosensors (Song, Xu, & Fan, 2006).

There are several different recognition elements in particular the proteins receptors, antibodies, antigens, enzymes, molecularly-imprinted polymers and nucleic acids. When the recognition component is an enzyme biosensor, the biosensor is identified as enzymatic. These biosensors were the first to show up and have as an advantage the fact that enzymes are highly specific/selective biological catalysts. There are however some disadvantages associated with these biosensors, in particular the reduced stability of these

materials of biological origin, changing its activity by varying pH and temperature (Choi, Kwak, & Park, 2010).

Biosensors utilizing antibodies as recognition elements are known as immunosensors. Typically, antibodies are bound to the transducer, and its interaction with the antigen is detected by various transducers. The great advantage of using antibodies relates to the high affinity and selectivity for these materials present in the target antigen, allowing a direct contact of a complex sample with a biosensor recognition surface. However, as enzymes, their performance varies with the external conditions of the environment (Perumal & Hashim, 2014).

Thus, recognition elements of synthetic origin that try to mimic the biological properties of materials have been assuming a growing role in the field of chemosensors. In essence, these materials must show a high affinity and selectivity for the target analyte or catalytic ability to transform also in a selective and sensitive way, a compound that is measurable by the transducer. A molecular recognition element may be connected to a different number of signal transducers (Perumal & Hashim, 2014). The transducer converts the electrical signal into a molecular or digital signal that can be measured, displayed, and analysed.

Transducers fall into four general categories, measuring electrochemical (namely amperometric and potentiometric), optical (ie, colorimetric, fluorescent, luminescent, and interferometry), mass (i.e., piezoelectric and acoustic waves) and calorimetric properties (temperature-based). The transducer of any biosensor must be able to adapt the recognition element, provide specificity for monitoring the measured property, and present high sensitivity for the current change in the concentration range of interest (Putzbach & Ronkainen, 2013).

#### *I.1.3.3.2. Electrochemical biosensors*

Electrochemical biosensors are the most common type of biosensors used today due to their portability, cost effectiveness, small size, and easy use. Electrochemical biosensors can be used at home or in the doctor's office as POC devices (Johnson & Mutharasan, 2014). Few electrochemical biosensors for the detection of miRNA have been developed so far, relying mostly on miRNAs labelled with electrocatalytic moieties or nanoparticle tags. However, such procedures need to enrich and label miRNAs, thereby increasing the

complexity of the operation. This has been justified by the low abundance of miRNAs in real samples (Labib & Berezovski, 2015).

The transduction element in electrochemical miRNA biosensors is often based in gold (Au) supports. Sensing is accomplished by measuring changes in the electrode or interfacial properties upon hybridization between the immobilized complementary DNA probe and the miRNA target. Nevertheless, label-free electrochemical biosensors have attracted attention in order to increase simplicity. They involve direct or catalysed oxidation of RNA bases, as redox reactions of reporter molecules, enzymes, and viral proteins recruited to the electrode surface by specific RNA probe-target interactions (Jamali, Pourhassan-Moghaddam, Dolatabadi, & Omidi, 2014).

Transduction in electrochemical miRNA biosensors has been investigated via amperometric, potentiometric, resistive and impedance-based approaches. Amperometric transducers rely on oxidation/reduction reactions, producing a current, which can then be measured. Amperometric-based biosensors for cancer detection that make use of specific DNA sequences as recognition element may turn out an extremely useful tool in cancer diagnosis. These sensors can detect the presence of gene mutations associated with cancer through recognition of and hybridization to specific DNA sequences, present in genomes of cancerous cells. Also, electrochemical biosensors offer the capability of detecting damaged DNA as well as the carcinogens that caused the damage (Tran et al., 2014). Electrochemical transducer technology is also widely used in immunoassays and protein arrays. Immunosensors, in which an antibody is coupled to an electrochemical transducer, have also been used for measuring cancer biomarkers. Several studies have been made combining miRNA and electrochemical biosensors, and these have been summarized in table I. Overall, most of the overall designs have been made in a complex way, aiming to ensure the good performance of the device.

**Table I:** Electrochemical biosensors for miRNA detection.

| Target miRNA | *Probe* | Brief Biosensing Mechanism | Nano-material | Technical arpproach | LOD | Linear Range | Ref. |
|---|---|---|---|---|---|---|---|
| *MIR-155* | — | Based on the conductive self-assembled multilayer comprised of Nafion, thionine (Thi) and Pd nanoparticles was successfully prepared. Nafion was firstly dropped on to a bare glass carbon electrode. Then Thi was absorbed by the cation exchanger Nafion. Pd nanoparticles layer which was used to immobilize target biomolecules was constructed by the amino group of Thi as linker. | GO[a)]/AuNP[b)] | GCE[c)]/DPV[d)] | 3.3 fM | 10 fM–1 nM | (Wu et al.,2014) |
| *MIR-155* | — | Magnetic bead/ligase chain reaction/T4 ligase. | PbS, CdS QD[e)] | GCE/SWV[f)] | 12 fM | 50 fM–30 pM | (Zhu et al., 2014) |
| *MIR-155* | — | Nafion/thionine/$H_2O$. The method is based on the combination of the high base-mismatch selectivity of ligase chain reaction (LCR) and the remarkable voltammetric signature of electrochemical QDs barcodes. Two reporting probes of RP1 and RP2 were labeled with PbS and CdS quantum dots to prepare PbS–RP1 and CdS–RP2 conjugates, and two capture probes of CP1 and CP2 were co-immobilized on magnetic beads (MBs) to fabricate MB–CP1CP2 conjugate. | Pd NP[g)] | GCE/CV[h)] | 1.87 pM | 5.6 pM–56μM | (Wu et al.,2013) |
| *MIR-155* | Oligonucleotide | This nanobiosensor is based on the glassy carbon electrode (GCE) with thiolated single stranded probe (SS-probe) and a novel electrochemical indicator; Oracet blue (OB), as an electrochemical biosensing system with the purpose of quantifying the plasma MIR-155 for early detection of the breast cancer. | GO/GNR[i)] | GCE[j)]/DPV[k)] and CV | 2 fM–8 pM | 0.6 fM | (Azimzadeh et al.,2015) |
| *MIR-155* | Oligonucleotide | The biosensor consists of (1) a thiolated (anti-MIR-155) immobilization on the gold screen printed electrode (Au-SPE), followed by the (2) non-specific binding blockage with mercaptosuccinic acid and (3) miRNA hybridization. | Au-SPE[l)] | CV; EIS[m)]; SWV | 10aM-100pM | 0.0057fM | This Work |

a) Graphene Oxide; b) Gold Nanoparticles; c) Glassy Carbon Electrode; d) Differential Pulse Voltammetry; e)Quantum Dots; f) Square Wave Voltammetry; g) Lead Nanoparticles; h) Cyclic Voltammetry; i) Gold Nanorods; j) Glassy Carbon Electrode; k) Differential pulse voltammetry; l) Gold Screen Print Electrodes; m) Electrochemical Impedance Spectroscopy;

**Table I:** Electrochemical biosensors for miRNA detection (Cont.).

| Target | Probe | Brief Biosensing Mechanism | Nano-material | Technical approach | LOD | Linear Range | Ref. |
|---|---|---|---|---|---|---|---|
| *MIR-24* | DNA | The synthetic DNA probes, being complementary with miRNA-24, were immobilized on MWCNT-modified glassy carbon electrodes by covalent cross-linking. Oxidation signal of guanine. | MWCNTs[a)] | GCE/DPV | 1pM | 1 pM–1nM | (Li et al., 2014) |
| *let-7c, let-7b* | PNA[b)] | A nanogapped microelectrode-based biosensor array. Capture probes immobilized in nanogaps of a pair of interdigitated microelectrodes and hybridization performed with their complementary target miRNA; the deposition of conducting polymer nanowires, nanowires, is carried out by an enzymatically catalyzed method. | Polyaniline nanowires | NW/SWV | 5.0fM | 10 fM -20 pM | (Fan et al. 2007) |
| *MIR-16* | LNA[c)] | Combining rolling circle amplification (RCA) | QD[d)] | AuSPE/ASV | 0.32 aM. | 10aM -10pM | (Wang, Hu, Zhou, Abdel-Halim, & Zhu, 2013) |
| *MIR-141* | DNA Aptamer | Label-free and reagentless microRNA sensor based on an interpenetrated network of carbon nanotubes and electroactive polymer. The nanostructured polymer film shows electroactivity in neutral aqueous medium in the cathodic potential domain from the quinone group embedded in the polymer backbone. | MWCNTs | GCE[e)]/CV[f)] and EIS[g)] | 8fM | 1 fM-100 pM | (Tran et al., 2013) |
| *MIR-21,* | PNA | TMB[h)]/HRP[i)]/Streptavidin-Poly-HRP80. DNA nanostructure-based interfacial engineering approach to enhance binding recognition at the gold electrode surface and improve the detection sensitivity | DNA TN[j)] | AuE[k)]/Amp[l)] | 10fM | 10fM-10nM | (Wen et al., 2012) |
| *MIR-122* | DNA | DNA Four-Way Junction/streptavidin | AuNP[m)] | SPCE[n)]/SWV[o)] | 2aM | 10 aM-1fM | (Labib, Khan, & Berezovski, 2015) |
| *MIR-21* | LNA | LNA molecular beacon/streptavidin-HRP/HQ. Reported DNA loading in gold nanoparticles and biotin multi-functionalized biobar codes, streptavidin–HRP was brought to the electrode through the specific interaction with biotin to catalyze the chemical oxidation of hydroquinone by $H_2O_2$ to form benzoquinone. | GO[p)]/AuNP | GCE/Amp | 0.06pM | 0.1pM–7pM | (Yin, Zhou, Chen, Zhu, & Ai, 2012) |

a)Multi-walled Carbon Nanotubes; b) Peptide Nucleic Acid; c) Locked Nucleic Acid; d) Quantum Dots; e) Glassy Carbon Electrode; f) Cyclic Voltammetry; g) Electrochemical Impedance Spectroscopy; h) Tetramethylbenzidine; i) Horseradish Peroxidase; j) DNA Tetrahedral Nanostructure; k) Gold Electrodes; l) Amperometry; m) Gold nanoparticles; n) Screen Printed Carbon Electrode; o) Square Wave Voltammetry; p) Graphene Oxide.

**Table I:** Electrochemical biosensors for miRNA detection (Cont.).

| Target | Probe | Brief Biosensing Mechanism | Nano-material | Technical approach | LOD | Linear Range | Ref. |
|---|---|---|---|---|---|---|---|
| *let-7c* | PNA | Peptide nucleic acid probe/polyaniline/$H_2O_2$ | $RuO_2NP$ | AuE/SWV | 2.0 fM | 5.0 fM–2. pM | (Peng, Yi, & Gao, 2010) |
| *MIR-21* | — | Star trigon structure/endonuclease/Methylene Blue. Glassy carbon electrode covered with gold nanoparticles (AuNPs) mediated by poly(diallyldi-methylammonium chloride). | AuNP | GCE/SWV | 30aM | 100 aM-1nM | (Miao, Wang, Yu, Zhao, & Tang, 2015) |
| *let-7b* | — | Nanoparticles catalyze oxidation of hydrazine. The nanoparticle tags, isoniazid-capped $OsO_2$nanoparticles, are brought to the electrode through a condensation reaction in order to chemically amplify the signal. | $OsO_2NP$ | ITO[q]/Amp | 80 fM | 0.30 pM-20 pM | (Gao & Yang, 2006) |
| *MIR-21* | DNA Aptamer | 3D DNA stem-loop probe/ferrocene. 3D DNA origami structure that enables electrochemical detection of lung cancer related microRNAs. The 3D DNA origami structure is constituted of a ferrocene-tagged DNA of stem-loop structure combined with a thiolated tetrahedron DNA nanostructure at the bottom. | Au NP/3D DNA | AuE/DPV[r] | 10pM | 100 pM-1µM | (S. Liu, Su, Li, & Ding, 2015) |
| *MIR-141* | DNA Aptamer | An electrochemical ELISA-like amplification strategy was set up using a secondary antibody conjugated to horseradish peroxidase (HRP). Hydroquinone is oxidized into benzoquinone by the HRP/$H_2O_2$ catalytic system. In turn, benzoquinone is electroreduced into hydroquinone at the electrode. The catalytic reduction current is related to HRP amount immobilized on the surface, which itself is related to miRNA. | MWCNTs/GO | SPGE[s]/SWV | 10fM | 10 fM–1nM | (Tran et al., 2014) |

q) Indium tin oxide; r) Differential pulse voltammetry; s) Screen-Printed Gold Electrode

### *I.1.3.3.3. Optical biosensors*

Optical biosensors are an alternative to electrochemical-based devices. These biosensors are light-based sensors that detect changes in specific wavelengths of light. The transducer can measure luminescence, fluorescence, colorimetry, or interferometry based phenomenon. The optical biosensors described so far in the literature for miRNAs have been summarized in table II.

Optical transducers convert changes in wavelengths in response to analyte recognition into an electrical/digital readout. Photonic crystal biosensors are a newly emerging class of biosensors that use an optical transducer. The photonic crystal sensor is designed to capture light from very small areas or volumes, allowing for greater sensitivity of measurement, and then transmitting that light into a high electromagnetic field for display. By measuring the light reflected by the crystal, this technique can detect when and where cells or molecules bind to or are removed from the crystal surface (Table II) (Jamali et al., 2014).

**Table II:** Optical biosensors for miRNA detection.

| Probe | Receptor | Target miRNA | Description | LOD (aM) | Linear Range (pM) | Ref. |
|---|---|---|---|---|---|---|
| DNA Aptamer | QD[a] | let-7a let-7b | miRNA binds directly with an enzymatic label of a peroxidase | 0.1aM | — | (Yan et al., 2013) |
| DNA Aptamer | GO[b] | MIR-16 MIR-21 MIR-26A | The capability of GO to discriminate ssDNA and double-stranded nucleic acid structure (DNA/DNA or DNA/RNA) in combination with the excellent organic fluorescence quenching of GO allowed the proposed strategy to detect miRNA in the same solution with high selectivity, while the ISDPR-based target amplification endows the detection method with high sensitivity | 2.1aM | — | (Dong et al., 2012) |
| Complementary Oligonucleotide | GO | MIR-126 | The method can also be used for rapid estimation of the miRNA-126 expressions in several different types of cells. | 3000aM | — | (Tu, Li, Wu, Zhang, & Cai, 2013) |

a) Quantum Dots; b) Graphene Oxide.

Analysing tables I and II, it can be seen that electrochemical miRNA biosensors have impressive sensitivity, ranging from attomolar (aM) - nanomolar (nM) levels. For example, an electrochemical biosensor with nano-structured electrode exhibited an LOD of 10aM. In addition to millimetre scale biosensor designs, nano-scale electrochemical miRNA biosensors are being currently investigated as potential miRNA biosensors (Bourigua, Maaref, Bessueille, & Renault, 2013). For example, nanowires and nanopores-based sensors displayed LODs of 1 fentomolar (fM) and 100 fM, respectively. The major

drawback of nano-scale devices is low sensor mass transfer rates which increases the time-to-results. Nano-scale biosensing approaches are promising as high sensitivity and multiplexing capability have been demonstrated.

However, LOD optical miRNA biosensors range from nM-fM levels. For example, surface plasmon resonance in silicon micro-rings has been investigated, displaying an LOD near 1nM, albeit with excellent signal-to-noise ratio suggesting higher sensitivity could be achieved (Koyun, Ahlatcıoğlu, & İpek, 2012).

### I.1.4. Objectives

The main goal of this thesis targets two different approaches. Firstly, a systematic review in order to identify the miRNAs related to breast cancer for subsequent application in biosensing device. The systematic revision was performed according to systematic reviews guidelines in order to establish the overall diagnostic accuracy of the measurement of miRNA in the diagnosis of breast cancer.

The second approach consisted in the development of a low cost sensing-platform based on thiolated anti-miRNA immobilization in a gold screen printed electrode (Au-SPE) surface for fast screening miRNA-155 in point-of-care.

# II. CHAPTER

## II.1. Experimental & Methods

### II.1.1. Systematic revision

The systematic revision approach is linked to the results presented in chapter III.2. The database used in order to determine most frequent miRNA targets *were PubMed*, MiRbase.org and PRISMA.org. Multiple Boolean descriptors associated to the Meshterus were used in this research work.

Each study was considered eligible for inclusion if it met the following inclusion criteria: (i) need to be published in *English*; (ii) include clinical trials on evaluation of miRNAs in the diagnosis of breast cancer; (iii) each group contained more than 10 patients. Finally, for data extraction the articles included author, publication year, state of study, diagnostic standard, patient number, specimen, test method, miRNA expression signature and methodological quality. The biological function of each miRNA was retrieved from MirBase.org.

### II.1.2. Biosensor assay

#### *II.1.2.1. Apparatus*

The electrochemical measurements were performed with a potentiostat/galvanostat from Metrohm Autolab, PGSTAT320N, controlled by NOVA 1.11 software. The Au-SPEs were purchased from DropSens (DS-C220AT). The Au-SPEs were interfaced in switch box from DropSens.

UV-Vis spectra (200-800 nm) were recorded on an Evolution 220 UV-visible spectrophotometer (Thermo Scientific) for obtaining the spectral data for each solution (Anti-miRNA155, miRNA-155; and mixture of both) and spectral range between 220 and 300 nm.

The morphological analysis of biosensor was performed by atomic force microscopy (AFM) in tapping mode. Surface characterization studies were conducted only on planar gold surfaces employed in regular surface plasmon resonance (SPR) measurements.
The measurements were conducted in tapping mode *(Veeco Metrology Multimode, Nanoscope IVA)*. The analysis of AFM images from tapping mode was obtained through *nanoscope software*.

### *II.1.2.2. Electrochemical Procedures*

All electrochemical studies, including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV) were performed in $5.0\times10^{-3}$ M of $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M of $[Fe(CN)_6]^{4-}$, prepared in PBS buffer pH 7.4, used as redox probe. All electrochemical assays were conducted in triplicate.

For CV assays, the potential was scanned from -0.5 to +0.5 V, at 50 mV/s. The EIS assays were performed at a formal potential of +0.14V, using a sinusoidal potential perturbation with amplitude 0.01V, and 50 frequency values, logarithmically distributed over a frequency range of 0.1 to 100000 Hz. The EIS data was fitted to a Randles equivalent circuit using 1.11 Nova Software from Autolab. In SWV studies, the potential range was from -0.2 to +0.8 V with a frequency of 25Hz and the step height of to 50mV.

The calibration curve/hybridization was performed for miRNA-155 concentrations ranging 1fM to 10nM prepared in Tris buffer, pH 7.4, and human blank synthetic serum (ranging from 0.00001fM to 10nM). The LOD was calculated as $\chi+3\sigma$, where $\chi$ was the average value of EIS or SWV of blank signals (obtained in the absence of miRNA-155) and $\sigma$ known standard deviation of EIS or SWV blank signals (Harvey, 2000).

Selectivity studies were conducted by competitive assay between miRNA-155 with two different species that can be found in biological fluids: BSA (0.30 µg/mL) and CA-15.3 (30 U/mL). All assays were conducted in triplicate.

# III. CHAPTER

## III.1. Results and Discussion

### III.1.1.Systematic revision

The first attempt to retrieve information from scientific evidences linking miRNA to breast cancer in literature made use of meta-analysis. Due to lack of data and non-response from many authors, most information was retrieved from a systematic review approach. This information was tabulated (Table I in section III.2.4) and discussed, describing clinical features, author, specimen, number of patients, testing method, targeted miRNAs, and biological features of each MIR. In this systematic review, only studies describing MIR-145, MIR-155 and MIR-181 were considered, due to their high frequency in most reported papers. Also, the analysis of miRNAs by means of quantitative reverse transcription PCR (qPCR) was included. The information combining the miRNA involved in breast cancer and its signalling pathways and genes was also tabulated and discussed. All these results are detailed next, in section III.2.4, containing the corresponding submitted paper.

### III.1.2. Biosensor assay

The construction of the biosensor relied in three steps. The first step consisted in covalent attach of the thiolated anti-miRNA155 probes on an Au-SPE surface. Next, the non-specific binding was blocked with mercaptossucinic acid (MSA). The third step consisted in hybridization with miRNA-155, by incubation on Au-SPE with different concentrations at 37ºC. The thiolated anti-miRNA155 probe had a complementary sequence to the target miRNA155.The chemical modifications of the Au-receptor surface were followed by electrochemical measurements as CV, EIS and SWV.

The overall performance of the biosensor was described in the section III.3. It includes calibration curves, selectivity study, and miRNA assays in human serum samples and in cell-line extracts.

The biosensor provided a sensitive detection of miRNA-155 from 10.0 aM to 1.0 nM with an LOD of 5.7 aM in human serum samples. Good results were obtained in terms of selectivity towards proteins present in human serum. The regeneration capability of the biosensor was evaluated and performed successfully. The last study consisted in miRNA assay with spiked samples of blank human serum. The sensor showed overall relative errors <10%.

The miRNA assay was performed also with cell-line extracts (cell line, MCF-7) that contained miRNA-155. MCF-7 breast cancer cell line is the most studied human breast cancer cell line in the world and is related with miRNA-155 (Lee, Oesterreich, & Davidson, 2015). The performance of the biosensor was tested also in extracts from melanoma cell-lines, acting as negative control (line cell without miRNA-155, B16F10).

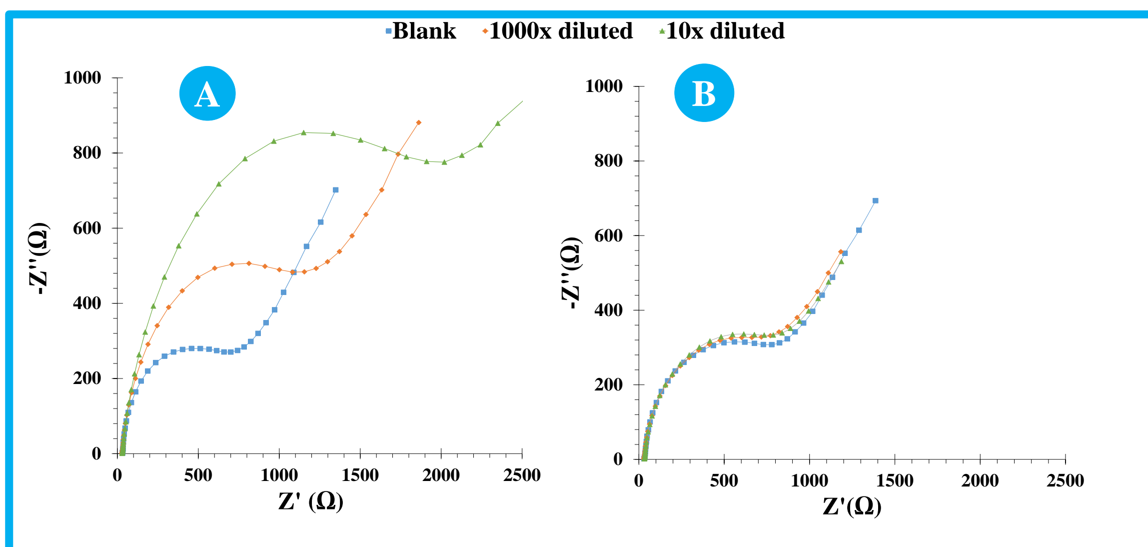


**Figure 3:** EIS measurements for different cell lines. (A) MCF-7 and (B) B16F10.

According to literature, the overexpression of miRNA-155 is linked to the cell growth in MCF-7 cell-line (Zhang, Zhao, & Deng, 2013). Different dilution degrees of the cell-line extracts were used, in order to evaluate the effect of the sample matrix on the sensor surface. Specifically, MCF-7 (Figure 3A) and B16F10 (Figure 3B) were tested for 100× and 1000× dilutions.

As can be seen in the Figure 3, only cell line with miRNA155 promoted an increase in the diameter of the semicircle of the Nyquist. This was due to an electron transfer resistance increase, caused by the hybridization of miRNA155 into its complementary oligonucleotide. Such hybridization generated an increase of the density of negative

charges over the sensory surface, coming from the phosphate backbone of the hybridized oligonucleotides, thereby hampering the surface electron transfer properties from a standard $Fe(CN)_6^{3-/4-}$ redox probe solution (S. Wu et al., 2015). No change in the diameter of the semicircle were observed with the negative control B16F10 (Figure 3B), meaning that the normal composition of cell extracts would not interfere, even if such extracts contain biomolecules expressed by other cancer cells besides breast cancer. These results evidenced the high selectivity of the biosensor towards miRNA155.

## III.2. Role of miR-145, miR-155 and miR-181a in Breast Cancer Systematic Review

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### III.2.1. Abstract

**Objective:** MicroRNAs have been report to be overexpressed in breast cancer patients. The aim of this systematic review is to identify which miRNAs are related to Breast Cancer.

**Methods**: The present study was performed according to Systematic Reviews guidelines in order to establish the overall diagnostic accuracy of the measurement of microRNA in the diagnosis of breast cancer. To identify studies eligible for systematic review in the PubMed citations.

**Results**: The present systematic review of 14 studies comprises 1897 patients available for analysis. Regarding the systematic review were included studies that addressed the three types of most described microRNAs in breast cancer (miRNA-155, miRNA-145, miRNA-181a). Also, has been described the microRNA involved in breast cancer and signaling pathways for each miRNA, and also genes that are involved in these miRNA.

**Conclusion:** MiRNA-155, 145 and 181a play an important role in breast cancer. These three miRNA are those that are more frequent and have more scientific evidence about their work, genes that act at the level of the carcinogenesis of breast cancer.

**Key words:** miRNA-155, miRNA-145, miRNA-181a, Breast cancer.

### III.2.2. Introduction

Breast cancer is a prevalent disease, accounting for significant morbidity and mortality with a worldwide incidence of over 1,400,000 women (Taplin et al., 2008). Breast cancer is the most frequent cancer among women and the second leading cause of cancer death in women in Europe and North America and its incidence is steadily increasing. Currently, mammography is the standard screening tool worldwide, nevertheless this technique is not absent of limitations and is associated with substantial over diagnosis as well as a high false-positive rate (Taplin et al., 2008). About 3% of the population screened annually with mammography will present abnormalities, but of those 65% will be false positives (Langagergaard et al., 2013). In addition, small cancers are easily missed, especially in younger women due to denser breast tissue. Therefore, there is a need for highly sensitive and specific minimally invasive biomarker based assays for early detection of breast cancer, alone or in combination with mammography. MicroRNAs (miRNA) have been recently describe as potential new diagnostic biomarkers not only for of breast cancer but also for other cancers (Langagergaard et al., 2013).

MiRNAs are small non-protein-coding RNA molecules, with a length of 18-25 nucleotides and act on the regulation of gene expression, and are fundamental in many cellular processes, such as differentiation, proliferation, apoptosis and metabolic homeostasis by modulating the expression of their target genes through cleaving mRNA molecules or inhibiting their translation (Jeffrey, 2008; Ryan, Robles, & Harris, 2010). Also control a wide range of pathological processes, including programmed cell death, oncogenesis and metastasis. The first step of biogenesis (Figure 1) of miRNA starts in nucleus with the gene transcription from RNA polymerase II, which produces a primary miRNA (Pri-miRNA). This transcript possesses a hairpin structure and is cleaved afterwards by Drosha (RNA polymerase 3) originating a precursor of mature miRNA called Pre-miRNA, with approximately 70 nucleotides. Exportin5 quickly exports pre-miRNA to cytosol. In the cytosol, the Pre-miRNA is processed by DICER, originating a double-ribbon miRNA with approximately 22 nucleotides. The previous product is then incorporated in a multimeric complex known as RISC (RNA-Induced Silence Complex). Within this complex, only one of the chains of miRNA will remain and it will be responsible for the post-transduction expression of target genes. Through the RISC complex we will obtain the pairing between the miRNA and the homolog target mRNA,

through reverse base complement. Therefore, when there is total pairing between both chains of RNA, RNA degradation occurs, but, on the other hand, when there is only partial pairing, translation's repression occurs followed by posterior degradation of mRNA (He & Hannon, 2004).

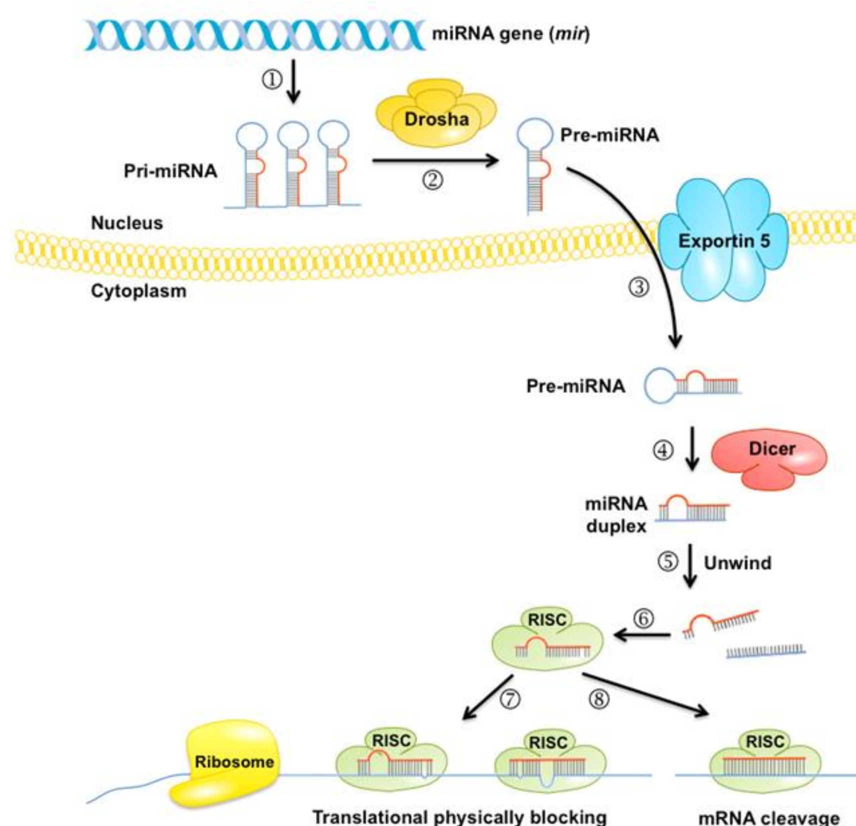


**Figure 4:** The current model for the biogenesis and post-transcriptional suppression of microRNAs. ① In nucleus, the miRNA gene is transcript from RNA polymerase II, which produces a primary miRNA, pri-microRNA (pri-miRNA). ②The pri-microRNA transcripts are first processed into ~70-nucleotide pre-miRNAs by Drosha inside the nucleus. ③ Pre-miRNA is quickly exported by Exportin 5 to cytosol. ④In cytoplasm the Pre-miRNA is processed by Dicer thus originating a double-ribbon miRNA.⑤This product is unwind then incorporated in complex RISC. ⑥ Through the RISC complex it will obtain the pairing between the miRNA and the homolog target mRNA, through reverse base complement. ⑦▪This subsequently acts on its target by translational repression or mRNA cleavage⑧, depending, at least in part, on the level of complementarity between the small RNA and its target.

Generally, a tumor suppressor miRNA typically blocks the expression of an oncogene mRNA. Conversely, miRNA oncogenes (also known as oncomir) block expression of tumor suppressor genes, leading to increased risk of tumor formation (Di Leva & Croce, 2010; Li et al., 2009; Wijnhoven, Michael, & Watson, 2007; Zhang, Pan, Cobb, & Anderson, 2007).

Aberrant expression levels of miRNAs have been observed in many solid tumors, including breast cancer. Furthermore, miRNAs and their targets appear to form a complex

regulation network. A single miRNA can regulate several mRNAs and a single mRNA may be targeted by a number of different miRNAs. Approximately one third of all human genes encoding proteins that are regulated by miRNA based computational predictions (J. Lu et al., 2005). Thus, measurement and quantification of the miRNA can translate in a more reliable method and less invasive for the presence of cancer. In addition, their expression provides information about the origin of the tumour and allows classifying the degree of tumour aggressiveness (Jeffrey, 2008; Ma, Teruya-Feldstein, & Weinberg, 2007; Zhang et al., 2007). One of the most important advantages of using circulating miRNAs as biomarkers, apart from being easily measured in blood samples, is their remarkable stability in plasma and serum, where they are most likely protected from RNase degradation due to their binding to Argonaut proteins (Arroyo et al., 2011; Mitchell et al., 2008). However, it remains unclear whether a particular combination of circulating miRNAs is superior to single miRNAs as a diagnostic test in early breast cancer. Different correlations have been described at very earlier stages between miRNA and several types of cancers, in which they present an abnormal expression levels. In breast cancer, the levels of expression of several miRNAs are significantly different between normal tissue and the tumour, and also between breast cancers with different molecular subtypes with a different prognosis, showing the different responses to endocrine therapy (Ma et al., 2007). As a result, we have proposed to perform a systematic review, in order to understand the relationship of miRNA and breast cancer. Furthermore, we propose to evaluate the diagnostic value of some miRNAs as biomarker.

## **III.2.3.**Material and methods

We performed a systematic review regarding the presence of particular type of miRNA and breast cancer.

### *III.2.3.1. Search strategy and study selection*

To identify studies eligible for systematic review, used citations in the PubMed, were surveyed combinations of several MeSH terms as “Breast cancer”, “miRNA” and “diagnosis”. A study was included if it met the following inclusion criteria: (i) published in English; (ii) e-clinical studies on evaluation of miRNAs in the diagnosis of breast cancer; (iv) each group contains more than 10 patients.

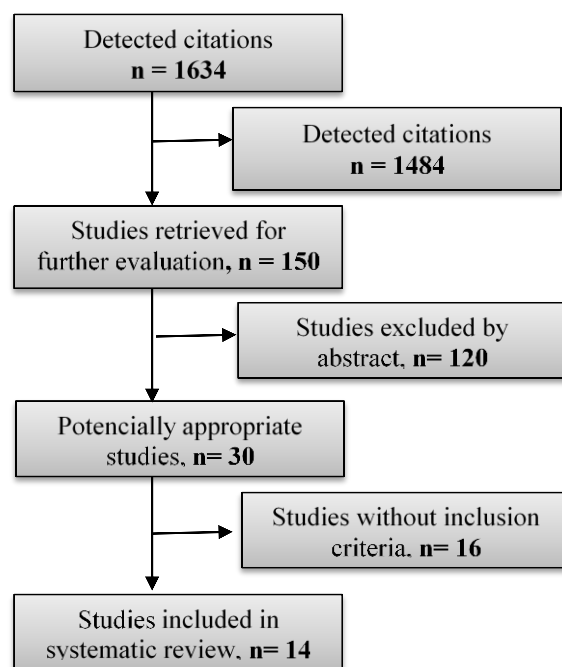


**Figure 5:** Flow chart of the search of systematic review.

#### *III.2.3.2. Data extraction*

Data retrieved from these articles included author, publication year, state of study, diagnostic standard, patient number, specimen, test method, miRNA expression signature and methodological quality.

#### *III.2.3.3. On-line databases*

To conclude the systematic reviews, as well assessing which are the most frequent miRNA target's, several online databases were used, namely PubMEd and Mirbase.org.

### **III.2.4.** Results

A table was developed to gather information more synthesized. Clinical characteristics of the works included were extracted the present study and the listed in Table I. In this table, the author, specimen, patients number, test method, the miRNAs that were analyzed, and finally, relevant results were reported for each study. In the present systematic review of 14 studies comprised 1897 patients available for analysis, however, only were considered studies describing miRNA-145, miRNA-155, and miRNA-181a due to their frequency on most studies. Also, miRNAs where analysed by means of quantitative reverse transcription PCR (qPCR).

**Table III:** Systematic review is of miRNAs related to Breast cancer.

| Specimen | No. | Method | miRNA | Revelant Results | Ref. |
|---|---|---|---|---|---|
| Seum | 42 | qPCR | miRNA-122<br>miRNA-375 | MiRNA-375 and miRNA-122, exhibited strong correlations with NCT (neoadjuvant chemotherapy) response and relapse with metastatic disease. Also, higher levels of circulating miRNA-122 specifically predicted metastatic recurrence in stage II-III BC (Breast Cancer) patients. | (Xiwei Wu et al., 2012) |
| Serum | 89 | qPCR | miRNA-10b<br>miRNA-34a<br>miRNA-141<br>**miRNA-155** | The significant increase of miRNA-10b, miRNA34a and miRNA-155 concentrations in the peripheral blood of breast cancer patients and the observed associations with tumor progression. Although the levels of miRNA-141 did not markedly change in the blood of patients with metastatic disease in comparison to patients with primary breast cancer. In tumor cell lines, the concentrations of the four miRNAs differed strikingly from those in the micrometastatic tumor cell lines. | (Roth et al., 2010) |
| Tissue, Serum | 84 | qPCR | miRNA-215<br>miRNA-299-5p<br>miRNA-411<br>miRNA-452 | Overexpression of miRNA-215, miRNA-299-5p, miRNA-411, and miRNA-452 in normal breast samples, in addition to the fact that miRNAs have a proven stability in blood samples. In other words, observe the reduction of miRNA expression was particularly obvious in serum samples from patients with untreated metastatic breast cancer. | (van Schooneveld et al., 2012) |
| Tissue, Serum | 183 | qPCR | miRNA-15a<br>miRNA-18a<br>miRNA-107<br>miRNA-133<br>miRNA-139<br>miRNA-143<br>**miRNA-145**<br>miRNA-365<br>miRNA-425 | Novel circulating 9-miRNA signature that discriminates between serum from early-stage, estrogen receptor (ER)-positive, breast cancer patients and healthy control subjects. Down-regulation of miRNA-145 in serum of women with breast cancer compared to normal controls, miRNA-133a and miRNA-143 were up-regulated, higher expression of miRNA-107 in breast cancer vs. normal tissue. Low levels of miRNA-139-5p and high levels of miRNA-425 were observed in breast cancer vs. adjacent normal tissue, miRNA-365 was found to be more highly expressed in breast cancer tissue. | (Kodahl et al., 2014) |
| Tissue, Serum | 46 | qPCR | miRNA-182 | MiRNA 182 was also overexpressed in the BC tissues compared with the para-carcinoma tissues. The serum levels of miR-182 in the ER-positive patients were considerably lower compared with those in the ER-negative patients. The serum levels of miRNA-182 in the progesterone receptor (PR)-positive patients were also found to be lower compared with those in the PR-negative patients. | (P.-Y. Wang et al., 2013) |
| Serum | 226 | qPCR | miRNA-195<br>let 7a<br>miRNA-10b | Elevated circulating miRNA-195 was found to be breast cancer specific and could differentiate breast cancer from other cancers and from controls with a sensitivity of 88% at a specificity of 91%. A combination of three circulating miRNAs, including miRNA-195, further enhanced the discriminative power of this test for breast cancer to 94%. | (Heneghan, Miller, Kelly, Newell, & |
| Serum | 103 | qPCR | **miRNA-155** | Showed that miRNA-155 was markedly increased in sera from breast cancer patients compared with normal controls. Showed a declined trend of miRNA-155 after surgery and chemotherapy, which raises the possibility to use it as an indicator for treatment response. | (Y. Sun et al., 2012) |

**Table III:** Systematic review is of miRNAs related to Breast cancer (cont.).

| Specimen | No. | Method | miRNA | Revelant Results | Ref. |
|---|---|---|---|---|---|
| Tissue, Plasma | 330 | qPCR | **miRNA-145** miRNA-451 | Showed that a combination of miRNA-145 and miRNA-451 was the best biomarker in discriminating breast cancer from healthy controls and all other types of cancers. MiRNA-145 have reduction of plasma level in breast cancer patients, in contrast miRNA-451 have elevation in plasma of breast cancer. | (Ng et al., 2013) |
| Serum | 110 | qPCR | miRNA-29a, **miRNA181a** miRNA-652 | MiRNA-29a, miRNA-181a, miRNA-652, profile of three circulating tumor-associated miRNA biomarkers for breast cancer are identified which in combination provide a sensitivity and specificity profile which exceeds that of several current clinical biomarkers. | (McDermott et al., 2014) |
| Serum | 30 | qPCR | miRNA-205 **miRNA-155** | MiRNA-205 was down-regulated in BC patient serum while miR-155 was up-regulated. High expression of miRNA-155 was associated with clinical stage, molecular type, Ki-67 and p53 in BC patients. Showed that ectopic expression of miRNA-205 significantly inhibits cell proliferation and promotes apoptosis. MiRNA-205 was down regulated and miRNA-155 was up-regulated in BC patient serum. MiRNA-155 was positive correlated with clinical stage and ki-67 and negatively correlated with p53 status. | (Liu et al., 2013) |
| Tissue, Plasma | 67 | qPCR | **miRNA-155** miRNA-31 | MiRNA-31, miRNA-155 expression in tissue samples was consistent with that in plasma samples, indicating that the miRNA-155 level is a direct reflection of miRNA-155 expression in cancerous tissues. | (Z. Lu et al., 2012) |
| Serum | 410 | qPCR | miRNA-18a **miRNA-181a** miRNA-222 | Overexpression of the 3 highest expressing miRNAs: miRNA-18a, miRNA-181a, and miRNA-222. | (Godfrey et al., 2013) |
| Tissue | 76 | qPCR | **miRNA-155** miRNA-125b **miRNA-145** miRNA-21 miRNA-155 | RNA expression could clearly separate normal versus cancer tissues, with the most significantly deregulated miRNAs being miRNA-125b, miRNA-145, miRNA-21, and miRNA-155. | (Iorio et al., 2005) |
| Tissue | 101 | Semiquantitative real-time PCR analyses | **miRNA-181a** | MiRNA-181a as a predictive biomarker for breast cancer metastasis and patient survival, and consequently, as a potential therapeutic target in metastatic breast cancer. MiRNA-181a expression was dramatically and selectively upregulated in metastatic breast tumors, particularly triple-negative breast cancers, and was highly predictive for decreased overall survival in human breast cancer patients. | (Taylor, Sossey-Alaoui, Thompson, Danielpour, & Schiemann, 2013) |

Once you know what the miRNA that appear more often, we prepare another table in order to know the miRNA involved in breast cancer and signalling pathways for each miRNA, and also genes that are involved in these miRNA.

#### *III.2.4.1. miRNA 145*

##### *III.2.4.1.1. Role of miRNA 145 in Breast Cancer*

MiRNA-145, this is known as a tumor suppressor in numerous human cancers, exhibits an inhibitory role in tumoral angiogenesis, cell growth and invasion and tumor growth through the post-transcriptional regulation of the novel targets N-RAS and VEGF-A, and may plays important inhibitory role in breast cancer malignancy by targeting these genes, early manifestation of altered. Studies showed that miRNA-145 is a putative tumor suppressor gene that is down regulated in several types of tumors and inhibits cell growth by targeting c-Myc and Insulin receptor substrate 1 (IRS-1). Furthermore, miRNA-145 is reported to target the pluripotency factors octamer-binding transcription factor 4 (OCT4), SOX2,( (SRY (Sex Determining Region Y)-Box 2) and KLF4 (Kruppel-like factor 4) and plays a key regulator in human stem cells (Singh & Mo, 2013).

##### *III.2.4.1.2. Role of miRNA 145 in other signaling pathways*

MiRNAs function to regulate the expression levels of other genes by a several mechanisms. Michael et al. (2003) subsequently verified expression of mir-hsa-145, and demonstrated significantly reduced levels of the miRNA in precancerous and neoplastic colorectal tissue. The mature sequence shown here represents the most commonly cloned form from large-scale cloning studies. Also, this miRNA is involved in the control of several groups of genes like transcription factors, coagulation factors, zinc finger CCCH-type containing 11A, gamma-aminobutyric acid A (GABA(A)) receptor-associated protein like 1, integrin, calmodulin, interferon gamma receptor 2 (Cui, Wang, & Chen, 2014; Michael, O'Connor, Pellekaan, Young, & James, 2003).

#### *III.2.4.2. miRNA 155*

##### *III.2.4.2.1. Role of miRNA 155 in Breast Cancer*

MiRNA-155 is overexpressed in breast cancer, thus being considered as an oncomir. Deregulation of their expression is involved in numerous pathological processes and oncogenic signaling. Also, showed that miRNA-155 was markedly increased in sera from breast cancer patients compared with normal controls. Furthermore, miRNA-155 expression was inversely correlated with ER and PR expression, regardless of human epidermal growth factor receptor 2 (HER-2) status. These findings suggest that miRNA-155 expression is deregulated in breast cancer and is involved in the oncogenesis of breast tumors. High expression of miRNA-155 was associated with clinical stage, molecular type, Ki-67 and tumor protein (p53) in breast cancer patients (Mattiske, Suetani, Neilsen, & Callen, 2012).

##### *III.2.4.2.2. Role of miRNA 155 in other signaling pathways*

MiRNA-155 plays an important role in various physiological and pathological processes. Exogenous molecular control *in vivo* of miR-155 expression may inhibit malignant growth, viral infections, and attenuate the progression of cardiovascular diseases. Also, miRNA-hsa-155 is regulates human HL-60 leukemia cells. MiRNA-hsa-155 resides in the non-coding BIC transcript (EMBL:AF402776), located on chromosome 21. Eis et al (2005) confirm that miRNA-155 is processed from the BIC transcript in human, and demonstrate elevated expression of miR-155 in lymphoma samples. The mature sequence shown here represents the most commonly cloned form from large-scale cloning studies. This miRNA is involved in the control of several groups of genes like protocadherin alpha, ATPases, transcription factors, RNA polymerases, hormones, and nucleases zinc finger transcription factors and zinc finger.

#### *III.2.4.3. MiRNA 181a*

##### *III.2.4.3.1. Role of miRNA 181a in Breast Cancer*

MiRNA-181, also an oncomir, is considered a predictive biomarker for breast cancer metastasis and patient survival. Consequently, mir-181a is a potential therapeutic target in metastatic breast cancer. MiRNA-181a expression was dramatically and selectively up regulated in metastatic breast tumors, particularly triple-negative breast cancers, and was highly predictive for decreased overall survival in human breast cancer patients. Another study showed that miR-181a functioned as a tumor suppressor by inducing apoptosis,

triggering growth inhibition and inhibiting invasion in glioma cells. If this is also the case in breast cancer, it raises the possibility that miRNA-181a could be exploited as a therapeutic intervention for breast (Bisso et al., 2013).

##### *III.2.4.3.2. Role of miRNA 181a in other signalling pathways*

This new class of genes has recently been shown to play a central role in malignant transformation. MiRNA are down regulated in many tumors and thus appear to function as tumor suppressor genes. The mature products miRNA-181a, miRNA-181b, miRNA-181c or miRNA-181d are thought to have regulatory roles at posttranscriptional level, through complementarity to target mRNAs. Furthermore, this miRNA is also involved in the control of many genes as transcription factors, ATases, importin 8, ataxia telangiectasia mutated, breast cancer 1 (BRCA1) associated protein, insulin-like growth factor 2 mRNA binding protein 2 (Hayes & Lewis-Wambi, 2015).

### **III.2.5.** Discussion

The diagnosis of breast cancer is crucial for implementing effective therapy so that the diagnostic tools should be as sensitive and specific as possible. Thus, the study of new types of biomarkers is essential in order to circumvent the shortcomings in the current diagnostic methods. Over the past years, several authors have been working with miRNA as possible biomarkers of breast cancer. Since dysfunction of miRNA in breast cancer was first reported in 2005, there have been many studies on the expression of several miRNAs and their roles in breast cancer. However, since late 2009, several groups reported on circulating miRNAs as markers for breast cancer detection. Most of these studies focused on comparing circulating levels of miRNAs in cancer patients to that of healthy controls, while only few studies investigated alterations in miRNA levels after surgery of early stage breast cancer (Kodahl et al., 2014). In current systematic review, we identified the three most frequent miRNA in breast cancer that are more investigated, namely, miRNA-155, 145 and 181a.

Regarding miRNA-155 is overexpressed in breast cancer, and deregulation of their expression involved in numerous pathological processes and oncogenic signaling. It was revealed that the miRNA-155 expression levels in breast cancer tissues were significantly elevated compared with those in paired normal tissues. Furthermore, miRNA-155 expression was inversely correlated with ER and PR expression, regardless of HER-2 status. These

findings suggest that miRNA-155 expression is deregulated in breast cancer and is involved in the oncogenesis of breast tumors (Kodahl et al., 2014; Liu et al., 2013).

Martin *et al* (2014) demonstrated that miR-155 expression increases tumorigenesis *in vivo* and determined miRNA-155 mediated transcriptome changes through next generation sequencing analysis. MiRNA-155 expression alters many signaling pathways, with the chief altered pathway being the mitogen-activated protein kinases (MAPK) signaling cascade and miR-155 induces shortening of target mRNA 3'UTRs and alternative isoform expression of MAPK related genes. In addition there is an observed increase in protein phosphorylation of components of MAPK signaling including extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and activator protein 1 (AP-1) complex members (Fra-1 and c-Fos) as well as elevated gene expression of MAPK regulated genes Zeb1, Snail, Plaur, and SerpinE1 (Martin et al., 2014).

Roth *et al* (2010) relate the significant increase of miRNA-10b, miRNA-34a and miRNA-155 concentrations in the peripheral blood of breast cancer patients and the observed associations with tumor progression. In primary and metastatic breast carcinomas, high transcript levels of miRNA10b were detected and associated with tumor progression. Overexpression of miRNA-10b in a non-metastatic breast cancer cell line induced invasion and metastasis. For example, knockdown of miRNA-34a by small interfering RNA significantly suppressed proliferation in the breast cancer cell line MCF-7, indicating that miR34a overexpression may be an acquired feature during carcinogenesis and support cell proliferation in breast tumors. Knockout mice, which do not express miRNA-155, showed that this miRNA played an important role in the immune system. In Hodgkin's lymphoma diffuse B cell lymphoma and breast cancer miRNA-155 was highly expressed (Roth et al., 2010).

Cai *et al* (2015) related that miRNA-155 has been implicated in the induction of breast, lung and liver cancer, but its role in prostate cancer has not been investigated. In this study, demonstrated that the expression of miRNA-155 was upregulated in prostate cancer tissues and cell lines as determined by quantitative reverse transcription-polymerase chain reaction. Furthermore, overexpression of miRNA-155 promoted cell proliferation, as indicated by MTT assay. Flow cytometric analysis demonstrated that inhibition of miR-155 induced cell cycle arrest and promoted apoptosis in prostate cancer cells. In addition, western blot analysis indicated that annexin 7(ANX7) was significantly downregulated in prostate cancer

tissues and cells. A luciferase reporter assay indicated that ANX7 was a target of miRNA-155, which suggested that miRNA-155 promoted the proliferation of prostate cancer cells by regulating ANX7 expression levels (Cai et al., 2015).

Another way analysis is Bioinformatics and metabolomics analysis. Kim *et al* (2015) used this technology showed a novel role of miRNA-155 in cancer metabolism through the up-regulation of thiamine in breast cancer cells. A bioinformatics analysis of miRNA array and metabolite-profiling data from NCI-60 cancer cell panel revealed thiamine as a metabolite positively correlated with the miRNA-155 expression level. They confirmed it in MCF7, MDA-MB-436 and two human primary breast cancer cells by showing reduced thiamine levels upon a knock-down of miRNA-155. To understand how the miR-155 controls thiamine level, a set of key molecules for thiamine homeostasis were further analyzed after the knockdown of miRNA-155. The results showed the expression of two thiamine transporter genes (SLC19A2, SLC25A19) as well as thiamine pyrophosphokinase-1 (TPK1) were decreased in both RNA and protein level in miRNA-155 dependent manner. Also, confirmed the finding by showing a positive correlation between miRNA-155 and thiamine level in 71 triple negative breast tumors and demonstrated a role of miRNA-155 in thiamine homeostasis and suggests a function of this oncogenic miRNA on breast cancer metabolism (Kim et al., 2015).

Sun *et al* (2012) suggest that serum miRNA-155 is a potential biomarker to discriminate breast cancer patients from healthy subjects. For the first time, demonstrated a declined trend of miRNA-155 after surgery and chemotherapy, which raises the possibility to use it as an indicator for treatment response (Y. Sun et al., 2012). Also another study, reveal that the effects of miRNAs on the development of breast cancer drug resistance. Among them, miR-155 takes part in a sequence of bioprocesses that contribute to the development of such drug resistance, including repression of forkhead box O3 (FOXO3), enhancement of epithelial-to-mesenchymal transition (EMT) and MAPK signalling, reduction of RhoA, and affecting the length of telomeres (Yu et al., 2015).

Liu *et al* (2012) used real-time PCR (qPCR) and analysed miRNA-205 and miRNA-155 in archived serum from 30 participants, 20 with breast cancer and 10 healthy people. MiRNA-205 was down regulated in breast cancer patient serum while miRNA-155 was up regulated. Furthermore, also analysed the relationship between the expression levels of these two miRNAs and the clinic pathologic parameters of breast cancer patients. High expression of

miRNA-155 was associated with clinical stage, molecular type, Ki-67 and p53 in breast cancer patients. By contrast, they found no significant correlation between miRNA-205 and breast cancer patient clinic pathologic parameters. Functional analysis showed that ectopic expression of miRNA-205 significantly inhibits cell proliferation and promotes apoptosis. MiRNA-205 was down regulated and miRNA-155 was up regulated in BC patient serum. MiRNA-155 was positive correlated with clinical stage and ki-67 and negatively correlated with p53 status (Liu et al., 2013).

Tsang *et al* (2015) used polymerase chain reaction (PCR)-based miR profiling was performed in a small mammary phyllodes tumours (PT) cohort to identify deregulated miRs in malignant PT. The purported roles and targets of these miRs were further validated. Unsupervised clustering of miRNA expression profiling segregated PT into different grades, implicating the miR profile in PT classification. Among the deregulated miRs, miR-21, miR-335 and miR-155 were validated to be higher in malignant than in lower-grade PT in the independent cohort by quantitative PCR (qPCR) ($P \leq 0.032$). Their expression correlated with some of the malignant histological features, including high stromal cellularity, nuclear pleomorphism and mitosis. Subsequent analysis of their downstream proteins, namely phosphatase and tensin homolog (PTEN) for miRNA-21/miRNA-155 and retinoblastoma (Rb) for miRNA-335, also showed an independent significant negative association between miRNA and protein expression (Tsang JY, 2015).

Lu *et al* (2012) purpose of the present study determined the tissue and plasma levels of miRNA-155 and miRNA-31 in 67 patients with invasive intra-ductal breast cancer and their correlation with the clinic pathological characteristics. Used a quantitative real-time-PCR (qPCR) assay, it was demonstrated that the plasma levels of miRNA-155 and miRNA-31 in patients were 6- and 5-fold higher than those in healthy individuals, respectively. In cancerous tissues, miRNA-155 expression levels were 5-fold higher compared with those in non-cancerous tissues, whereas no difference was observed with miRNA-31 expression. The expression levels of miRNA-155, but not miRNA-31, were inversely correlated with ER and progesterone receptor PR expression. The tissue and plasma levels of miRNA-155 and miRNA-31 were not correlated with HER-2 expression levels. Furthermore, high levels of plasma miRNA-155 and miRNA-31 were identified in the tumors of TNM stage II. Also, miRNA-155 was mainly expressed in patients with a pathology score of 3 for ER or PR expression; miR-31 expression was higher in patients with a pathology score of 2. These results suggest that miRNA-155 and miRNA-31 are differentially expressed in breast cancer

patients. Their correlation with the clinic pathological characteristics may aid the diagnosis and treatment of invasive intra-ductal breast cancer (J. Lu et al., 2005; Z. Lu et al., 2012).

Recent studies indicate that a series of isomiRs can be yielded from a miRNA locus, and these physiological miRNA isoforms have versatile roles in miRNA biogenesis, so the Wu *et al* (2015), performed a comprehensive analysis of miRNAs at the miRNA and isomiR levels in Breast Cancer using next-generation sequencing data from The Cancer Genome Atlas (TCGA). Abnormally expressed miRNA (miRNA-21, miRNA-221, miRNA-155, miRNA-30e and miRNA-25) and isomiR profiles could be obtained at the miRNA and isomiR levels, and similar biological roles could be detected. IsomiR expression profiles should be further concerned, and especially isomiRs are actual regulatory molecules in the miRNA-mRNA regulatory networks. The study provides a comprehensive expression analysis at the miRNA and isomiR levels in Breast Cancer, which indicates biological roles of isomiRs (Wu et al., 2015)

Igglezou *et al* (2014) measured RNA levels of three microRNAs with tumorigenic or angiogenic potential (miRNA-155, miRNA-195, and miRNA-21) in blood samples taken from patients with early breast cancer, both preoperatively and postoperatively. The results that showed elevated postoperative levels of miRNA-195 were detected only in patients who developed early tumor relapse and that miRNA-155 levels tended to increase three days postoperatively and fell below baseline one month post-surgery ($p<0.05$). The results of this pilot study indicate a possible involvement of miRNA-155 in surgery-induced angiogenesis and potential prognostic significance of high postoperative levels of circulating miR-195 in patients with breast cancer (Igglezou, Vareli, Georgiou, Sainis, & Briasoulis, 2014)

Another miRNA that is very involved in signaling pathways in breast cancer is miRNA-145, this is known as a tumor suppressor in numerous human cancers, exhibits an inhibitory role in tumoral angiogenesis, cell growth and invasion and tumor growth through the post-transcriptional regulation of the novel targets N-RAS and vascular endothelial growth factor A (VEGF-A), and may plays important inhibitory role in breast cancer malignancy by targeting these genes, early manifestation of altered. Also this is miRNA that is a putative tumor suppressive gene that is down-regulated in several types of tumor and inhibits cell growth by targeting c-Myc and IRS-1.

Furthermore, miRNA-145 is reported to target the pluripotency factors OCT4, SOX2, and KLF4 and plays a key regulator in human stem cells. MiRNA-145 expression in atypical

hyperplasia and carcinoma in situ lesions in breast cancer suggests that this miRNA may have a potential clinical application as a novel biomarker for early detection (Ng et al., 2013; van Schooneveld et al., 2012).

Muthiah *et al* (2015), delivery of therapeutic miRNA using nanoparticles enhances the chances of successful delivery and expression of genes at the target site. They used polysorbitol-mediated transporter (PSMT) in the cellular delivery of miRNA-145. The polysorbitol backbone possesses osmotic properties and leads to enhanced cellular uptake. PSMT delivers genes into cells by a caveolae-mediated endocytic pathway. Caveolae expression is usually altered in transformed cancer cells. Also, confirmed that miRNA-145 delivered with PSMT has reduced the proliferation of cancer cells in in vitro efficiently when compared to control carrier. *In vivo* efficiency of the PSMT/miRNA-145 is to be confirmed and then move forward towards further steps in cancer treatment (Muthiah et al., 2015).

Ekin *et al* (2015) used nanotechnology has the potential to facilitate the detection, diagnosis, and treatment of cancer cases. Successful delivery of nucleic acids into cancer cells with the use of nanoparticles would be a significant improvement for medical and cellular biology. The use of nanoparticle-based vehicles in clinical treatment is considerably important for treating genetic disorders. Gold nanoparticles (AuNPs) have been suggested as therapeutic delivery tools for cancer. Because miRNAs, which induce post-transcriptional gene silencing, are deregulated in cancer cells, they are also considered as strong candidates for cancer therapy applications. In prostate and breast cancer, miRNA-145, a well-known tumor suppressor miRNA, is strongly downregulated in tumor tissues compared to their corresponding normal tissues, and demonstrate the efficient of delivery of miR-145 to prostate/breast cancer cells. Also, they showed that delivery was more efficient when the AuNP-RNA-miRNA carrier complex was formed at an elevated temperature of 72 °C (Ekin, Karatas, Culha, & Ozen, 2014).

Ng *et al* (2013) identified significant elevation of the miRNA-16, miRNA-21, and miRNA-451 and significant reduction of miRNA-145 in the plasma of breast cancer patients. The combination of plasma miRNA-145 and miRNA-451 levels provided the best marker for breast cancer prediction. Also showed that the best combination of miRNA-451 and miRNA-145 plasma levels are very specific to breast cancer and did not elevate in all other cancer types included in this study. Besides, elevation of plasma miRNAs has been detected in not only advanced stages of breast cancer but also early stages (pre-invasive stages) of breast

cancer such as ductal carcinoma *in situ* (DCIS) suggested that this marker might be useful for early diagnosis. Studies showed that miRNA-145 is a putative tumor suppressive gene that is down regulated in several types of tumors and inhibits cell growth by targeting c-Myc and IRS-1. Furthermore, miRNA-145 is reported to target the pluripotency factors OCT4, SOX2, and KLF4 and plays a key regulator in human stem cells (Ng et al., 2013). Recently, Sachdeva *et al* (2010) (Sachdeva & Mo, 2010) further characterized miRNA-145 in different cancer cell lines and found that miRNA-145 functions as a tumor suppressor in a cell type–specific manner. This report showed that miRNA-145 is a tumor suppressor affecting invasion and metastasis (van Schooneveld et al., 2012).

Iorio *et al* (2014) compared normal breast tissue with human breast cancer. MiRNAs are also aberrantly expressed in human breast cancer. The overall miRNA expression could clearly separate normal versus cancer tissues, with the most significantly deregulated miRNAs being miRNA-125b, miRNA-145, miRNA-21, and miRNA-155. Results were confirmed by microarray and Northern blot analyses. It possible identify miRNAs whose expression was correlated with specific breast cancer biopathologic features, such as estrogen and progesterone receptor expression, tumor stage, vascular invasion, or proliferation index (Iorio et al., 2005).

Sun *et al* (2014) compared to the normal control, a total of 21, 47, and 107 differentially expressed miRNAs were screened in DCIS, invasive and metastatic breast cancer, respectively. Specific differentially expressed miRNAs of the three subtypes were identified, including hsa-miRNA-99a and hsa-miRNA-151-3p for DCIS breast cancer, hsa-miRNA-145 and hsa-miRNA-210 for invasive breast cancer, and has-miRNA-205 and has-miRNA-361-5p metastatic breast cancer. Furthermore, 220, 43, 446, 307, 587 and 328 interaction pairs of the specific miRNA targets were predicted. Multiple GO functions and KEGG pathways were enriched with the miRNA targets and their interacted genes. Also, screened the most representative miRNAs of the three different subtypes of breast cancer, which may act as the putative markers in the diagnosis of different subtypes of breast cancer (E. H. Sun et al., 2014).

Finally, miRNA 181a levels were up regulated in breast cancer patients compared with healthy controls, for that and therefore, this miRNA is considered to be a potential marker for breast cancer. MiRNA-181a may be useful in detecting certain carcinomas at early stages, particularly when the individuals are clinically asymptomatic for extended periods.

Also, another studies indicated the precise targets of miRNA-181a in breast cancer remain elusive, and it is unclear how miRNA-181a may be involved in tumor formation or how the levels relate to prognosis. MiRNA-181a expression level was lower in early stage breast cancer, leading to speculation that the link between K-ras and miRNA-181a may play a role in the etiology of this malignancy. Another study showed that miRNA-181a functioned as a tumor suppressor by inducing apoptosis, triggering growth inhibition and inhibiting invasion in glioma cells. If this is also the case in breast cancer, it raises the possibility that miRNA-181a could be exploited as a therapeutic intervention for breast (Guo & Zhang, 2012).

McDermott *et al* identified four miRNAs (miRNA-29a, miRNA-181a, miRNA-223 and miRNA-652) with deregulated expression in the circulation of women with Luminal A-like breast cancer. MiRNA-181a and miRNA-652 were down regulated in Luminal A-like breast tumor tissue, while miRNA-29a was not. These findings support the hypothesis that circulating miRNA expression profiles may not act as a direct window on tumor activity and brings into question the mechanism by which they enter the blood stream, in addition to their functional role, if any, in the peripheral circulation. Also, miRNA-181a has previously been reported as being significantly under-expressed in the serum of women with breast cancer compared to healthy controls (McDermott et al., 2014).

Godfrey *et al* (2013) showed miRNA-181a is believed to target the tumor suppressor gene programmed cell death protein 4 (PDCD4), which inhibits tumor neoplastic transformation. In breast cancer cell lines, miRNA-222 overexpression has been reported to be associated with tamoxifen resistance through targeting the cell cycle inhibitor p27 (Kip1). Also, they found two recent case control studies have provided evidence that both miRNA-222 and miRNA-181a are overexpressed in the serum of patients with breast cancer. One used sequencing by oligonucleotide ligation and detection (SOLiD) of serum samples obtained prior to surgery from 13 breast cancer cases compared with samples from 10 healthy controls and found 26 miRNAs that were overexpressed in cases, including miRNA-222 and miRNA-181a (Godfrey et al., 2013).

Taylor *et al* (2013) established one relationship with miRNA-181a and transforming growth factor β (TGF-β)–regulated "metastamir" that enhanced the metastatic potential of breast cancers by promoting epithelial-mesenchymal transition, migratory, and invasive phenotypes. Mechanistically, inactivation of miRNA-181a elevated the expression of the pro-apoptotic molecule Bim, which sensitized metastatic cells to anoikis. Along these lines,

miRNA-181a expression was essential in driving pulmonary micrometastatic outgrowth and enhancing the lethality of late-stage mammary tumors in mice. Finally, miRNA-181a expression was dramatically and selectively up regulated in metastatic breast tumors, particularly triple-negative breast cancers, and was highly predictive for decreased overall survival in human breast cancer patients. Collectively, their findings strongly implicate miRNA-181a as a predictive biomarker for breast cancer metastasis and patient survival, and consequently, as a potential therapeutic target in metastatic breast cancer (Taylor et al., 2013).

### III.2.6.Conclusion

The present systematic review suggests an important role of miRNA miRNA-155/145/181a in breast cancer. These three miRNA are those that are more frequent and have more scientific evidence about their work, genes that act at the level of the carcinogenesis of breast cancer.

Regardless of the great number of studies in this field, performing additional research to clarify the role of miRNA in breast cancer seems critical, needed and potentially precious. This situation assumes an even bigger importance due to the fact that the current diagnostic tool for breast cancer, mammography is not totally reliable. Therefore, developing new potential biomarkers is essential, in a way that combining various methodologies will allow having a more preventing way to confirm breast cancer and thus, apply a better treatment to the patient.

However, to prove that these miRNA are those with greater sensitivity and specificity needed to do a meta-analysis, could not be confirmed, as the authors did not make their data available for this study.

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## III.3. A novel and simple approach for monitoring attomolar concentrations of miRNA-155 in Breast Cancer

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### III.3.1. Abstract

This work, describes for the first time, a simple biosensing design to yield an ultrasensitive electrochemical biosensor for a cancer biomarker detection, miRNA-155, with linear response down to the attomolar range. MiRNA-155 was selected for being overexpressed in breast cancer.

The biosensor was assembled in two stages: (1) the immobilization of the anti-miRNA-155 that was thiol modified on an Au-screen printed electrode (Au-SPE), followed by (2) blocking the areas of non-specific binding with mercaptosuccinic acid. The hybridization between anti-miRNA-155 and miRNA-155 yielded changes in the electrical properties of the biosensor. Atomic force microscopy (AFM) and electrochemical techniques including cyclic voltammetry (CV), impedance spectroscopy (EIS) and square wave voltammetry (SWV) confirmed the surface modification of these devices and their ability to hybridize successfully and stably with miRNA-155.

The final biosensor provided a sensitive detection of miRNA-155 from 10.0 aM to 1nM with a low detection limit (LOD) of 5.7 aM in real human serum samples. Good results were obtained in terms of selectivity towards breast cancer antigen CA-15.3 and bovine serum albumin (BSA) proteins, which are commonly present in human serum from breast cancer patients in relatively high amounts. Raw fluid extracts from cell-lines of melanoma did not affect the biosensor response (no significant change of the blank), while raw extracts from breast cancer yielded a positive signal against miRNA-155, as expected.

This simple and sensitive strategy is a promising alternative for simultaneous quantitative analysis of multiple miRNA in physiological fluids for biomedical research and point-of-care (POC) diagnosis.

**Keywords:** Breast cancer; Anti-miRNA-155; miRNA-155; Biosensors, Eletrochemistry**.**

### III.3.2. Introduction

Cancer is a public health concern worldwide. One in three women and one in two men will develop cancer during lifetime in developed countries (Siegel et al., 2012). Early detection is the only known approach that may improve these indicators, but traditional diagnostic tools for breast cancer detection are mostly ineffective, including: i) clinical and physical examinations, ii) imaging mammography, iv) ultrasound magnetic resonance imaging and v) histopathology. Clinical and physical examination have been shown insufficient, driving patients into mammography or ultrasound studies. In turn, mammography has limited sensitivity, yielding a high rate of false positive results. This approach may also lead to an accumulated exposure to radiation, which is considered an additional and significant risk factor (Yahalom et al., 2013). Ultrasound is a non-invasive and safe tool, but it cannot replace mammograms, especially in women above 40, and is unable to screen many of the cancers. Thus, there is urgent need to develop non-invasive, simple and low risk methods for screening/diagnosing breast cancer. Considering the high prevalence of cancer diseases and the high frequency of analysis to be performed in clinical context, there is a strong need that such test are made available in POC point-of-care (POC) (Riaz et al., 2013). (Grieshaber et al., 2008).

Currently, strong efforts are being developed to monitor serum biomarkers as early detection of cancer. The measurement of a panel of such biomarkers is considered an economic and minimally invasive diagnostic assay, able to provide safe information about the presence or absence of the disease, as well as its joint evolution to other clinical and pathological approaches (Mirabelli & Incoronato, 2013) (Chung et al., 2014).

In breast cancer, the most prevalent serum markers used in clinical practice are carcinoembryonic antigen (CEA), carbohydrate antigen 15-3 (CA 15-3), circulating cytokeratins such as tissue polypeptide antigen (TPA), tissue polypeptide specific antigen (TPS) and cytokeratin 19 fragment (CIFRA-21-1), and the proteolytically cleaved ectodomain of the human epidermal growth factor receptor 2 (s-HER2). The most widely used serum biomarker is CA 15-3, however, due to its lack of sensitivity at an early stage of the disease, it cannot be used exclusively. In addition, this marker can lead to false results after initiation of treatment without clinical correlation. Also, other biomarkers like TPA and CEA are less sensitive than CA-15.3 (Sun et al., 2012).

But recent technological advances have led to the identification of a new class of biomarkers named miRNAs that may lead to a novel strategy in early cancer screening (He et al., 2015; Volinia et al., 2012). MiRNAs contain 18-24 nucleotides in length (Johnson & Mutharasan, 2014) and play an essential role in biological processes such as development, cellular proliferation, apoptosis and response to stress, and tumorigenesis. Aberrant expression levels of miRNAs have been observed in many solid tumours, including breast cancer (Fu et al., 2011), accounting their dysregulated expression in cancer. In addition, miRNAs are stable in the blood and have high/low expressions that can be correlated to chemo-resistance, (Kong et al., 2010) (Tavallaie et al., 2015).

In breast cancer, several studies have supported an abnormal expression of miRNA-155 (MIR-155) in patients with the disease (Mattiske et al., 2012). The overexpression of MIR-155 was considered as a breast cancer risk factor (Zeng et al., 2014), being associated with clinical-pathological markers, tumour subtype, poor survival rates, metastasis events and invasive properties of breast cancer, as well as high tumour grade, advanced stage and lymph node metastasis (Mattiske et al., 2012). MIR-155 is involved in controlling several mechanisms of cell survival, cell growth, radio/chemo-resistance (Liu et al., 2015) (Mattiske et al., 2012) (Liu et al., 2015), inhibiting target genes such as FOXO3A, RhoA, and SOCS1. The use of miRNA-155 as a potential biomarker in breast cancer opens the possibility towards a simple serological test for prognosis/diagnosis and follow-up of breast cancer under therapy.

Biosensors are today a successful route towards POC testing, allowing fast results and a direct sample reading, without the need for transporting samples into the laboratory (Campuzano et al., 2014) (Labib and Berezovski, 2015) (Bohunicky and Mousa, 2011). Devices relying in electrochemical transduction are currently the most common type of biosensors. This is an outcome of their portability, simplicity, sensitivity, low cost, small size, rapidity of response, ease of use, and possibility of reading samples directly over a wide range of concentrations (Xia and Zhang, 2014). In general, these features of electrochemical biosensors depend of the recognition element immobilized on the transducer, which is responsible for interacting with the analyte in a selective mode (Choi et al., 2010) (Grieshaber et al., 2008).

In electrochemical miRNA detection, the recognition elements are mostly linear oligonucleotide strands of DNA/RNA nature with which the miRNA target hybridizes (Catuogno et al., 2011; Johnson & Mutharasan, 2014; Tavallaie, De Almeida, & Gooding,

2015). The transduction event measures the changes in the electrode or interfacial properties occurring upon hybridization, by means of electrochemically-active reporter species, including small redox molecules (such as ferric species, guanine oxidation) or enzyme-substrate pairs (such as horseradish peroxidase/hydrogen peroxide) (Johnson & Mutharasan, 2014). Concurrent methods are mostly devoted to biosensors using other transduction modes (optical or electromechanical) or molecular-based approaches, including polymerase amplification, microarray (coupled to amplification approaches), spectroscopy, sequencing, cloning, and Northern blotting (Johnson & Mutharasan, 2014). Biosensors with other modes of transduction are typically linked to higher detection levels and molecular based approaches involve complex operations that are either expensive or far from POC requirements.

Regarding the specific electrochemical determination of miRNA-155, there is a limited number of works reported in the literature. The first one was presented by (X. Wu, Chai, Yuan, Su, & Han, 2013), combining a conductive composition of Nafion, thionine (Thi) and Pd nanoparticles, displaying electrocatalytic activity for $H_2O_2$, and providing an LOD of ~1.9 pM. Zhu et al., 2014, combined high base-mismatch selectivity of ligase chain reaction with reporting probes labeled with two different quantum dots (Zhu, Su, Gao, Dai, & Zou, 2014). This elegant and complex approach had an LOD of 12-31 fM. Later on, Wu et al. 2015 presented another biosensor based on catalyzed hairpin assembly target recycling and cascade enzymatic electrocatalysis for signal amplification, in a quite complex approach, to yield an LOD of 0.35 fM (S. Wu et al., 2015). Hu et al., 2015 presented an horseradish peroxidase and graphene quantum dots combination, this time giving rise to a 0.14 fM LOD (Hu, Zhang, Wen, Zhang, & Wang, 2015). Very recently, Azimzadeh et al. 2015, has proposed the modification of glassy carbon electrode surface by a thiolated probe-functionalized with gold nanorods and decorated on the graphene oxide material, having as reporter label Oracet Blue. The LOD was higher than the previously reported, equal to 0.6 fM (Azimzadeh, Rahaie, Nasirizadeh, Ashtari, & Naderi-Manesh, 2015). Overall, the previous attempts of producing a sensitive device for miRNA detection have been effective but quite complex in terms of biosensor assembly and no significant improvements in terms of analytical features have been gained within time.

Thus, a simple and low cost concurrent approach would be appreciated, especially if a direct reading of serum samples is rendered possible and if the biosensor assembly is set to a minimum complexity, trying to carefully optimize critical variables, instead. Thus, the main

goal of this work is the development of an alternative approach that may lead to a sensitive and selective electrochemical biosensing of miRNA-155 in breast cancer, and yielding better analytical features than those reported so far. The biorecognition element used herein is the complementary oligonucleotide of synthetic origin (anti-miRNA-155) that was simply immobilized on Au Screen printed electrodes (Au-SPE). Non-specific binding was blocked with mercaptosuccinic acid modification, for subsequent hybridization of the anti-MIR with the target analyte, MIR-155. All studies regarding the chemical modification and optimization of the biosensor design, electrical characterization, and analytical application are presented herein.

## **III.3.3.**Experimental section

### *III.3.3.1. Apparatus*

The electrochemical measurements were performed with a potentiostat/galvanostat from Metrohm Autolab, PGSTAT320N, controlled by NOVA 1.11 software. The gold-screen printed electrodes (Au-SPEs) were purchased from DropSens (DS-C220AT), built with (i) a counter electrode made of gold; (ii) a reference electrode and electrical contacts made of silver; and (iii) a gold working electrode with 4 mm diameter. Also, the Au-SPEs were interfaced in switch box from DropSens, allowing reading electrochemical assays.

UV/Vis studies were made in the spectrophotometer Evolution 220 UV-visible from Thermo Scientific. AFM studies were made in a Nanoscope IVA from Veeco Metrology Multimode.

### *III.3.3.2. Reagents*

The water used in this work was ultrapure Mili-Q laboratory grade (conductivity <0.1µS/cm). In specific assays, the water was autoclaved with 0.1% diethylpyrocarbonate (DEPC) in order to increase the MIR stability by decreasing RNase activity.

The chemical reagents used along this work were used without further purification. Potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate II ($K_4[Fe(CN)_6]$) trihydrate, magnesium chloride (MgCl), and sodium phosphate ($Na_2HPO_4$), all obtained from Riedel-deHaen; Serum cornay human was purchased from PZ CORMAY S.A., Poland; Phosphate buffer saline (PBS) and bovine serum albumin (BSA) were from Amresco; Calcium chloride (CaCl) and potassium chloride (KCl) from Merck;

Ethylenediamine tetraacetic acid (EDTA) from BDH; Sodium chloride (NaCl) and absolute ethanol (99.5%) from Panreac; Hydroxymethyl-aminomethane (Tris) from Fisher BioReagents; Sodium citrate ($Na_3C_6H_5O_7$) from Analar Normapur; Sulphuric acid ($H_2SO_4$), dithiothreitol (DTT), mercaptosuccinic acid (MSA, 97%), DEPC, and cancer antigen 15-3 (CA-15.3) from Sigma.

The oligonucleotide probes were Anti-miRNA-155 (oligonucleotide) and miRNA-155, purified by HPLC and obtained from the Metabion. The sequences are as follows: anti-miRNA-155 (anti-miRNA155), 5'-HS-AAA AAA AAC CCC UAU CAC GAU UAG CAU UAA-3'; miRNA-155 (miRNA-155), 5'-UUA AUG CUA AUC GUG AUA GGG GU-3'.

#### *III.3.3.3. Solutions*

All solutions were prepared in ultrapure water, autoclaved with 0.1% DEPC. The stock solution of oligonucleotide (1.16 µg/ml, Anti-miRNA) was prepared in SSPE buffer containing 0.02 M EDTA, 2.98 M NaCl and 0.2 M phosphate buffer (20× diluted, pH 7.4).

The stock solution of 0.6 µg/mL of miRNA-155 was prepared in Tris-HCl, containing 0.02 M Tris, 0.14 M NaCl, 0.001 M $MgCl_2$, 0.005 M KCl and 0.001 M $CaCl_2$ (pH 7.4). Less concentrated standards were obtained by accurate dilution of the previous solution in 0.01 M Tris buffer containing 0.001 M EDTA and 0.05 M NaCl (pH 7.4). The immobilization and hybridization buffer were prepared in the some buffer solution. The buffer for regeneration (SSC) contained 3.0 M NaCl and 0.3 M trisodium citrate (0.1× diluted, pH 7.4).

Solutions of interfering species were prepared in the same buffer used in the immobilization and hybridization stages. The concentration of miRNA-155 was 0.6 µg/ml and the interfering species were BSA (0.30µg/ml) and CA-15.3 (30U/mL). The miRNA-155 solutions were prepared in real human serum (Cornay Human Serum), 1000× diluted. The electrochemical studies were made in a solution of $5.0\times10^{-3}$ M of $K_3[Fe(CN)_6]$ and $5.0\times10^{-3}$ M of $K_4[Fe(CN)_6]$ redox probe, prepared in PBS buffer, pH 7.4.

#### *III.3.3.4. Preparation of electrochemical biosensor on Au-SPE*

Before modification, the working Au-SPE surface was cleaned by washing with absolute ethanol, followed by CV electrochemical treatment with 0.5 M $H_2SO_4$, for 10 cycles (500

mV/s; -0.1 to 1.5V). Finally, the Au surface was washed with ultrapure water in order to eliminate chemical compounds.

The synthesis of the biosensor was made in two steps. Step (1) consisted in incubating anti-miRNA155 (1µM/mL) with DTT (0.1M) on the Au-SPE working area. The probe solution was prepared in buffer, pH 7.4 that contained Tris, EDTA, NaCl, previously heated at 90ºC for 5 minutes, and incubated for 2h at room temperature. The electrode surface was then washed with ultrapure water several times. In step (2) the non-specific binding areas were blocked by incubation the sensory surface with 0.002 µg/mL MSA, for 2h, at room temperature. The hybridization of the probe with the target miRNA-155 was made with several miRNA-155 standards solutions, prepared in buffer or in blank sera. Each standard was incubated for 30 minutes at 37ºC.

#### *III.3.3.5. UV Characterization*

The hybridization stage occurring between probe and target miRNA was followed out of the biosensor format by UV-Vis spectrophotometry. It was done by measuring the spectral data of each individual solution (Anti-miRNA155, miRNA-155; and mixture of both) in the spectral range from 220 to 300 nm.

#### *III.3.3.6. AFM analysis*

The morphological analysis of biosensor was performed by AFM in tapping mode. All surface characterization studies were conducted on planar Au surfaces employed in regular surface plasmon resonance (SPR) measurements. For this, anti-miRNA-155, Anti-miRNA-155/ MSA and anti-miRNA-155/MCA/miRNA155 materials were assembled on SPR planar gold chips following the same procedure as that described for the Au-SPEs. The analysis of AFM images were collected and handled by the nanoscope software, coupled to the same equipment.

#### *III.3.3.7. Electrochemical Procedures*

All electrochemical assays were conducted in triplicate. CV assays was made for scanning potentials from -0.5 to +0.5 V, at a scan-rate of 50 mV/s. EIS assays were performed at an open circuit potential, using a sinusoidal potential perturbation with an amplitude of 0.01V, and 50 data points, logarithmically distributed over 0.1 to 100000 Hz frequency range. The

EIS data was fitted into a Randles equivalent circuit using 1.11 Nova Software from Autolab. SWV assays were conducted for a potential range from -0.2 to +0.8 V, with a frequency of 25 Hz and a step height of 50mV.

The electrical properties of modified surfaces were followed-up by CV, EIS and SWV assays, performed in a redox probe solution of $5.0\times10^{-3}$ M of $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M of $[Fe(CN)_6]^{4-}$, prepared in PBS buffer, pH 7.4. Calibration curves made use of EIS and SWV assays and included standard solutions of miRNA-155, prepared in Tris buffer pH 7.4 (ranging from 1.0 fM to 10 nM), or in human serum (ranging from 0.01 aM to 10 nM). The LOD was calculated as X+3σ, where X was the average value of EIS or SWV for blank signals (obtained in the absence of miRNA-155) and σ is the known standard deviation of EIS or SWV for the blank's signal (Harvey, 2000).

Selectivity studies were conducted by competitive assay between miRNA-155 with two different biomolecules that can be found in biological fluids, such as BSA (0.30 µg/mL) and CA-15.3 (30 U/mL).

#### *III.3.3.8. MiRNA-155 assay*

The performance of the biosensor was analysed by the standard addition method, in order to determine the miRNA-155 concentration in spiked human blank serum. MiRNA-155 concentrations were set to different levels, equal to 0.1 pM and 100 pM, always lying within the linear range of the device. The human serum was diluted in TRIS buffer. All the assays were conducted in duplicate and analytical data was collected in EIS.

### **III.3.4.**Results and Discussion

#### *III.3.4.1. Biosensor assembly*

The schematic construction of the miRNA-155 biosensor is shown in Figure 1, and involved three main stages: (1) Au-SPE pre-treatment; (2) oligonucleotide immobilization and (3) non-specific binding blockage. The miRNA-155 hybridization assay was the final approach made with the biosensor, and concerned the analytical stage.

Initially, Au-SPEs were cleaned first by washing with ethanol, two times, and after by electrochemical cleaning, making use of consecutive CV assays in a $H_2SO_4$ solution. This oxidative stage allowed the oxidation of any organic species allocated on the surface and

improved the electrochemical properties of the bare Au electrode, by decreasing the peak-to-peak separation of the Fe redox probe and increasing peak currents.

The probe was then covalently bond to the clean SPE by incubating the Au working electrode in a solution of anti-miRNA-155 with a thiol group at its 5'end, in a hydrated chamber, at room temperature, and for a set time period. (Figure 6A). The probe had several consecutive adenine nucleotides between the –SH end and the complementary sequence to miRNA-155, acting as spacer and conferring mobility to the probe (in order to reduce steric hindrance at the hybridization stage). Before incubation, the anti-miRNA-155 probe was treated with 0.10M DTT for 15 minutes, in order to disrupt disulphide bonds, and warmed at 90ºC for 5 minutes to ensure that the strand had a linear pattern. This last heating stage was found crucial, as without it the amount of immobilized probe was much smaller (less than 10%) and irreproducible.

Next, the non-specific binding was blocked with MSA (Figure 6B). This compound has an –SH bond allowing its covalent binding to free Au areas existing on the working electrode. This stage was fundamental to avoid the direct interaction between any biomolecule in a sample and the gold.

The analytical event involved the hybridization between the probe and miRNA-155. It was made by incubating the working electrode area with different concentrations of target miRNA (Figure 6C). The different standard solutions were incubated on the working Au-SPE surface at 37ºC, for a set amount of time.

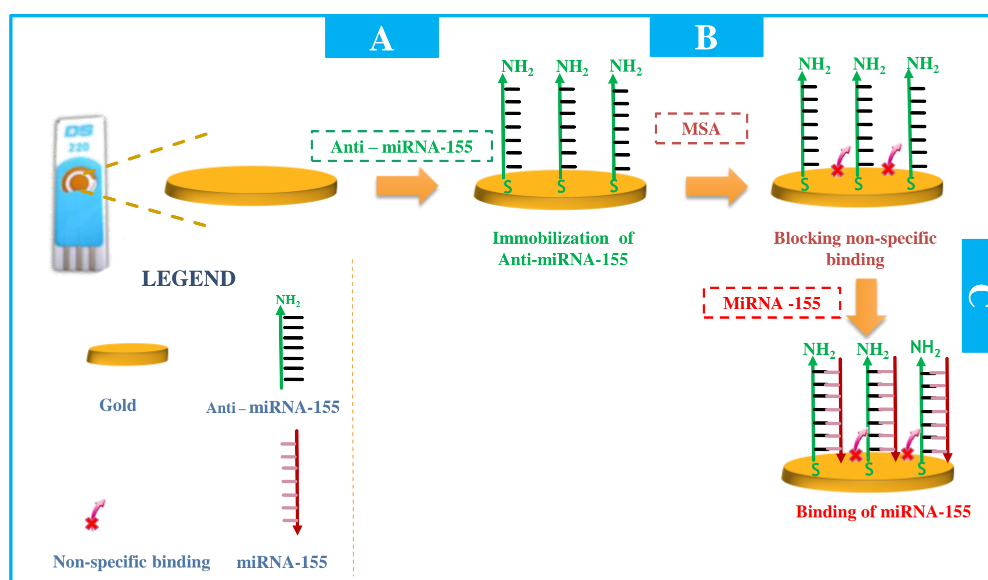


**Figure 6**: Schematic illustration of electrochemical biosensor for detection of miRNA-155. (A) Immobilization of Anti-miRNA-155 on gold surface; (B) Blocking the non-specific binding; and (C) Hybridization with miRNA-155.

#### *III.3.4.2. Morphological characterization of the biosensor*

The morphological features resulting from each modification stage were studied on planar gold surfaces, previously employed in regular SPR measurements and cleaned by oxidative solution. This was a fundamental strategy, as the Au layer on the SPEs was deposited by printing approaches and therefore its surface was too rough to allow detecting any morphological change promoted by a single monolayer modification.

Thus, the study was made on three different assemblies: clean Au-SPR, Au-SPR/Anti-miRNA155 and Au-SPR/Anti-miRNA-155/miRNA-155 (Figure 7), prepared as described for the SPEs. The first image shows the typical observation of the clean gold surface (Figure 7A). This surface was not as flat as expected, because the Au-SPR had been reused in previous experiments. Still, its roughness was low enough to allow detecting changes related to the subsequent chemical modification.

Indeed, the immobilization of the Anti-miRNA-155 on the clean Au-SPE rendered significant changes (Figure 7B), both in surface roughness (2.84 nm) and deepness (127.50 nm). The observed surface showed more roughness and deepness compared with the clean gold (1.39 nm and 17.57 nm, respectively), which was consistent with the addition of a miRNA monolayer onto the Au-SPE surface.

Finally, the miRNA-155 hybridization on the Au-SPR/Anti-miRNA155 surface produced greater morphological changes (Figure 7C). Both roughness and deepness decreased due to the hybridization event, thereby confirming that the complementary oligonucleotide sequences hybridized. The formation of an apparently more flat surface could be justified by the presence of stable double strand oligonucleotide formations of negative charge, distributed all around the surface and all of the same length; the strong negative outer surface in each double strand formation would contribute to repel each other and create an image of a homogenous surface.

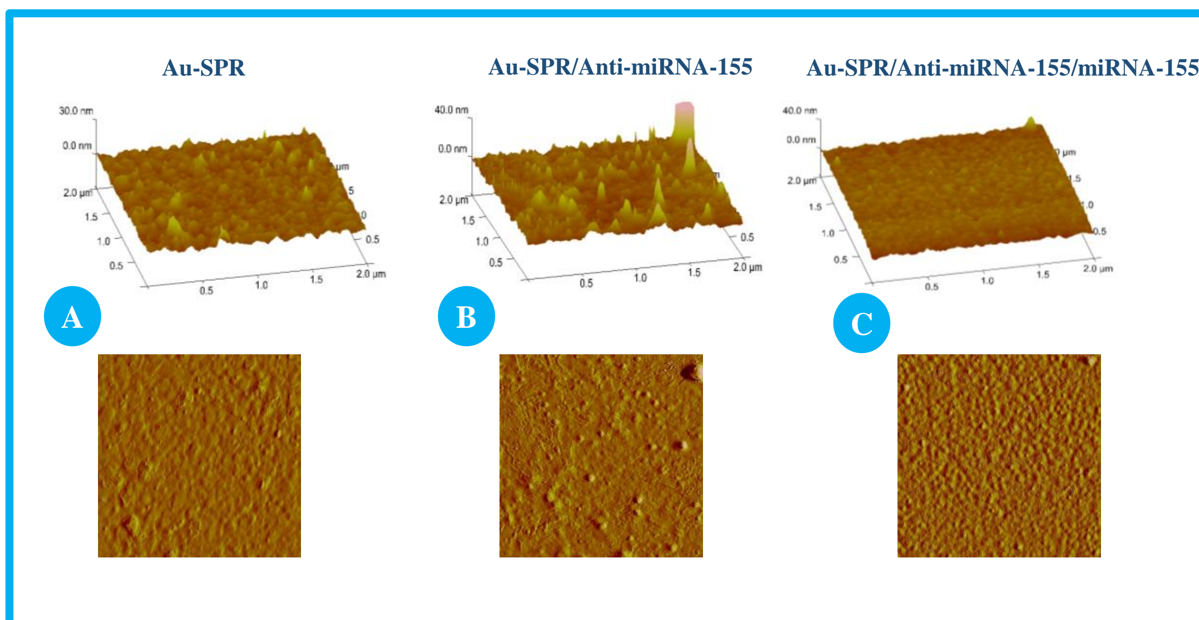


**Figure 7:** AFM images in 2D (below) and 3D (on top) views of different materials. (A) Au-SPR (B) Au-SPR/Anti-miRNA155 and (C) Au-SPR/Anti-miRNA-155/miRNA-155.

#### *III.3.4.3. UV Characterization*

After making the morphological characterization of the surface, UV/Vis absorption spectroscopy was carried out, in order to confirm that the reaction between Anti-miRNA-155 and miRNA-155 had occurred. This was done because, according to the literature, nucleic acids, nucleosides, and nucleotides absorb strongly UV light, and the sum of their individual nitrogenous bases generates the overall absorbance in the UV spectrum of DNA and RNA (*RNA Nanotechnology*, 2014). The results obtained are shown in Figure 8.

The UV spectra of individual solutions of anti-miRNA-155 and mirRNA155 had a single peak, around ~290 nm (Figure 8A and B). This confirmed the existence of oligonucleotide species in single-strand formation. According to literature, the molecules responsible for the absorbance of radiation at this wavelength are the aromatics nitrogenous bases (adenine and uracil; cytosine and guanine) (*RNA Nanotechnology*, 2014).

After mixing both solutions (Figure 8C), the spectra showed no peaks. During this stage, hybridization led to nitrogenous base pairing, resulting in the absence of absorbance by the solution. This lack of the absorption upon hybridization was led by a hypsochromic shift, as stacking of nitrogenous bases in native miRNA-155 interfered with UV absorption. Thus, these results have proven that the hybridization process had successfully occurred between anti-miRNA-155 and miRNA-155 single strands in the test solution.

Moreover, when the hybridized strands were separated by heat, the peak of absorbance of light was recovered. This phenomenon corresponded to subsequent bathochromic shift (*RNA Nanotechnology*, 2014) and confirmed the possibility of reversing the hybridization stage.

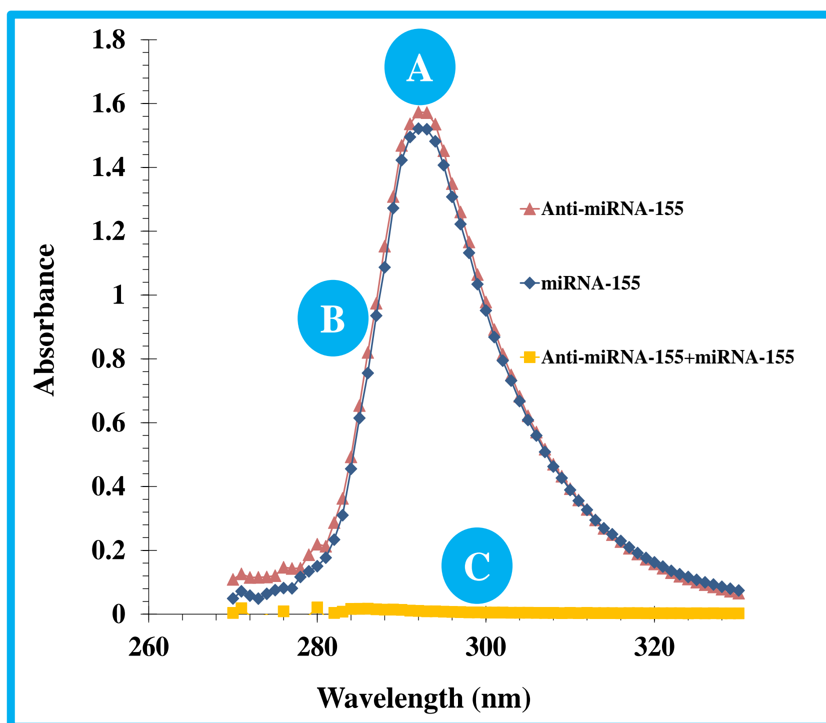


**Figure 8**: UV/Vis absorption spectroscopy of the Anti-miRNA-155 (A) and miRNA-155 (B) and respective complex.

### *III.3.4.4. Electrochemical control of the gold surface modifications*

The chemical modification of the Au-SPE surface produced alterations in its electrical features, evaluated by monitoring the electron transfer properties of a standard redox system. Different redox systems were tested for this purpose (Ruthenium and Iron), prepared in different buffer conditions. A redox probe of $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ was selected, at pH 7.4, as the electrical changes monitored by these conditions generated more sensitive alterations at the detected signal. The electrochemical approaches used for this purpose were CV, EIS and SWV assays.

CV analysis is shown in Figure 9A. When compared to the clean surface, the Au-SPE modified with anti-miRNA and MSA showed increased peak-to-peak potential separation and decreased cathodic/anodic peak currents. This accounted an increased charge-transfer resistance at the Au-SPE surface after each modification stage. After hybridization with miRNA-155 (Au-SPE/Anti-miRNA-155/miRNA-155), the peak current decreased more,

and a slight shift was observed in the peak potentials, thereby confirming the occurrence of chemical changes.

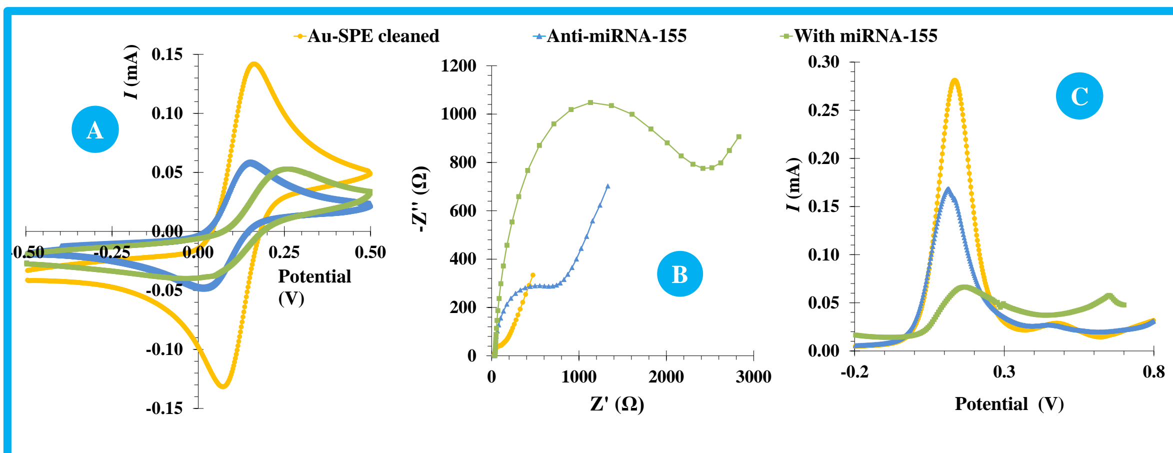


**Figure 9:** Electrochemical assays for controlling the gold surface modification, in $5.0{\times}10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0{\times}10^{-3}$ M $[Fe(CN)_6]^{4-}$, solution, prepared in in buffer (TRIS with EDTA and NaCl). CV (A), EIS (B) and SWV (C) assays.

The corresponding EIS measurements are shown in Figure 10 and were consistent with CV data. Randle's equivalent circuit was used to fit the physico-chemical process occurring at the gold electrode surface and EIS spectra were represented as Nyquist plots – including a semicircle, in which the diameter corresponded to the electron transfer resistance ($R_{ct}$) (Panagopoulou, Stergiou, Roussis, & Prodromidis, 2010) and the linear part represented the diffusion limited process (Suni, 2008). The Randle's equivalent circuit included several elements, in which the high frequency region is dominated by the double layer capacitance (Cdl) and the magnitude of the electrolyte solution resistance (Rs) (X. Liu, Duckworth, & Wong, 2010); the charge-transfer resistance ($R_{ct}$), which is inversely proportional to the rate of electron transfer; and the Warburg diffusion element (W*)* at higher frequencies, accounting for the diffusion of ions. Overall, the bare gold electrode showed a straight line or very small semicircle domain, which suggested a mass diffusion limiting step of the electron transfer process. After, the immobilization of thiol-based materials on the clean Au-SPE, the diameter of the semicircle of the Nyquist plot increased considerably. This observation was consistent with an increased electron transfer resistance at the surface, enhanced by the fact that the single strand anti-miRNA155 was negatively charged through its phosphate backbone, thereby hampering the electrode transfer event of a negatively charged redox couple (S. Wu et al., 2015). The hybridization of the probe with miRNA-155 also increased the Rct values, thereby confirming its effect upon the electron transfer and the increased density of negative charges occurring at the surface.

SWV data is presented in Figure 9C and is also consistent with EIS and CV assays. As it is more sensitive than CV, the changes in electrical transfer properties of the several modification stages were more evident in SWV. Voltammograms indicated a decrease in peak current for assembly and hybridization events.

Overall, CV, EIS and SWV assays were consistent, and confirmed the occurrence of chemical changes at the gold surface, both at the biosensor assembly and at the hybridization event. From a practical point of view, EIS and SWV techniques seemed more sensitive to the hybridization event, being therefore selected for the subsequent analytical application.

#### *III.3.4.5. Analytical Performance of miRNA sensor*

The analytical performance of the biosensor was evaluated by calibration curves in EIS and SVW measurements. For this, increasing concentrations of miRNA-155 were incubated at the surface for a specific amount of time, and the resulting $R_{ct}$ (in EIS) or current intensity ($I$, in SWV) values obtained with the iron redox probe measured against logarithm of miRNA-155 concentration. It is important to highlight that several variables were evaluated and optimized, including concentration, temperature and time given for the incubation of anti-miRNA solution on the clean gold surface, time and temperature given for the hybridization stage, buffer composition, etc.. The analytical data presented next regards only the best conditions selected.

In general, EIS (Figure 10A) measurements showed that increasing concentrations of miRNA-155 increased the electrical resistance of the surface, as the diameter of the semicircles in the Nyquist plots increased. A linear plot was observed between logarithm Rct and logarithm of miRNA-155 concentration. The response of the biosensor in EIS was tested in three calibrations, made with independent sensors, in different days, and was found highly reproducible. All these showed a linear trend between 1.0 fM and 100 pM.  As may be seen in Figure 10B, the second and third calibrations had an average slope of 0.086 logΩ/decade, with a standard of deviation 2.2 %. The squared correlation coefficient of all calibrations was always >0.99, and the average limit of detection was 0.54 fM.

SWV assays also showed linear plots, but this time between current intensity ($I$, in SWV) and logarithm of miRNA-155 concentration. The linear behaviour was observed from 1.0 fM and 10 nM (Figure 10C). The increasing miRNA-155 concentrations were revealed by the decreasing peak currents of redox probe solution. The following calibrations are presented in Figure 3D, showing average slopes of 25.18 µA/decade and square correlation

coefficients >0.99. The standard of deviation of the these assays was ~0,5% (Figure 3D), and the limit of detection was 2.8 aM.

Overall, the assembled biosensor showed consistent, sensitive and reproducible calibration curves, both EIS and SWV assays. The obtained results suggested that the biosensor displayed a highly selective and sensitive response against miRAN-155.

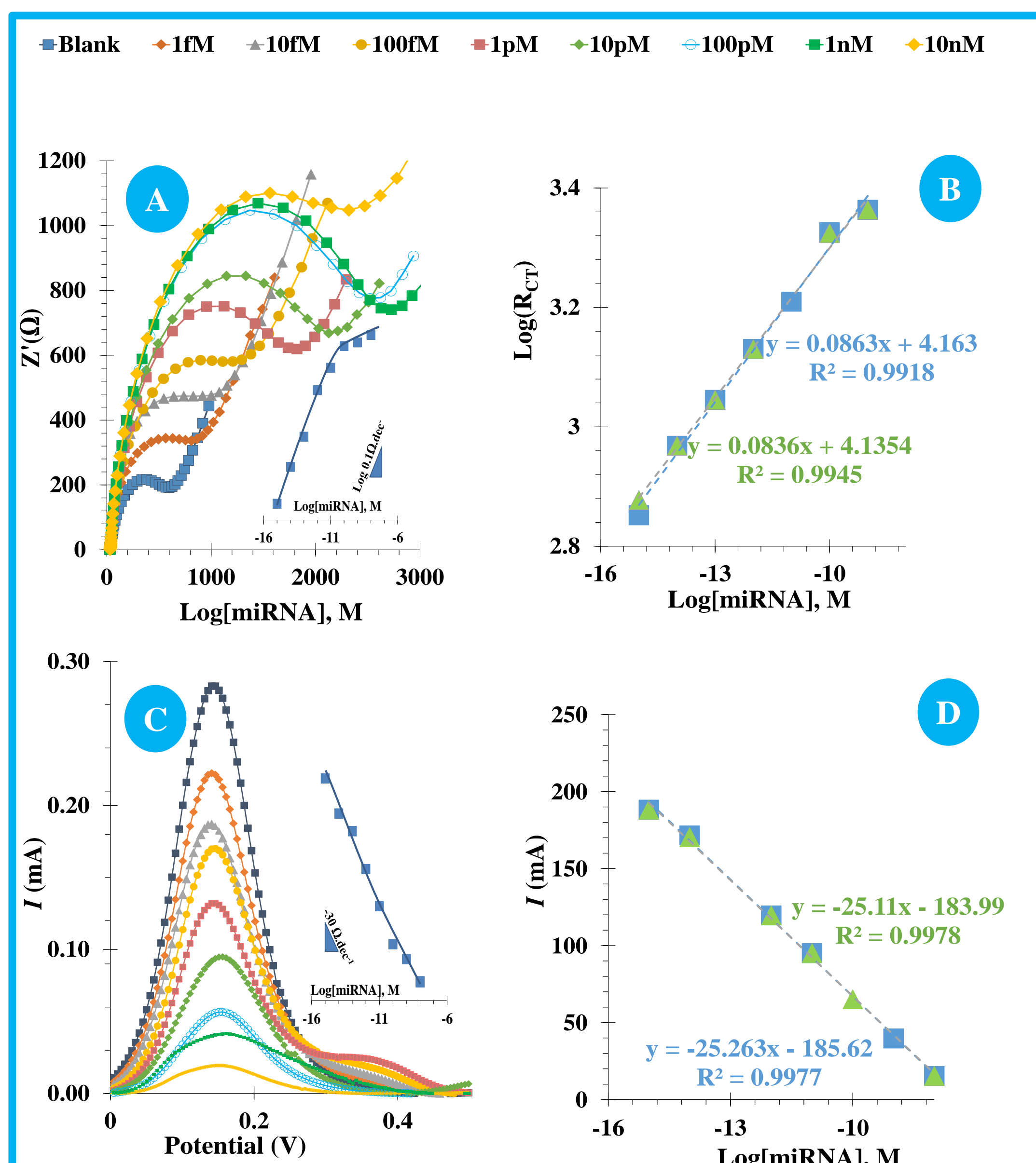


**Figure 10**: EIS(A) and SWV(C) measurements, and the corresponding calibration curves (B e D), in $5.0\times10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M $[Fe(CN)_6]^{4-}$, in buffer (TRIS with EDTA and NaCl), pH 7.4, with different concentration of miRNA-155.

### *III.3.4.6. Regeneration of Au-SPE*

The regeneration of a biosensor after a calibration assay maybe achieved by several physical (enthalpic interaction, entropic interactions, thermal regeneration-temperature) and chemical (acid/base-mediated regeneration, detergents, glycine, urea and another buffers) approaches. In this work, physical (temperature) and chemical (buffer treatment) assays were tried out (Goode, Rushworth, & Millner, 2014) and the results presented in Figure 11.

The temperature was considered as an important parameter to be tested herein since the 3D structure of oligonucleotides is typically affected by temperature changes. A temperature increase leads to increased kinetic energy of the molecules, which allows binding forces to be overcome after reaching the suitable temperature. The first assay was made by heating the Au-SPE up to 90ºC, the same temperature that ensured previously that the anti-miRNA-155 was linear and free to hybridize. The 90 ºC is indeed the typical denaturation temperature used in PCR (Polymerase chain reaction) assays, for which no irreversible denaturing or decoupling of oligonucleotide base pairs would be expected (Champaign, 2013).

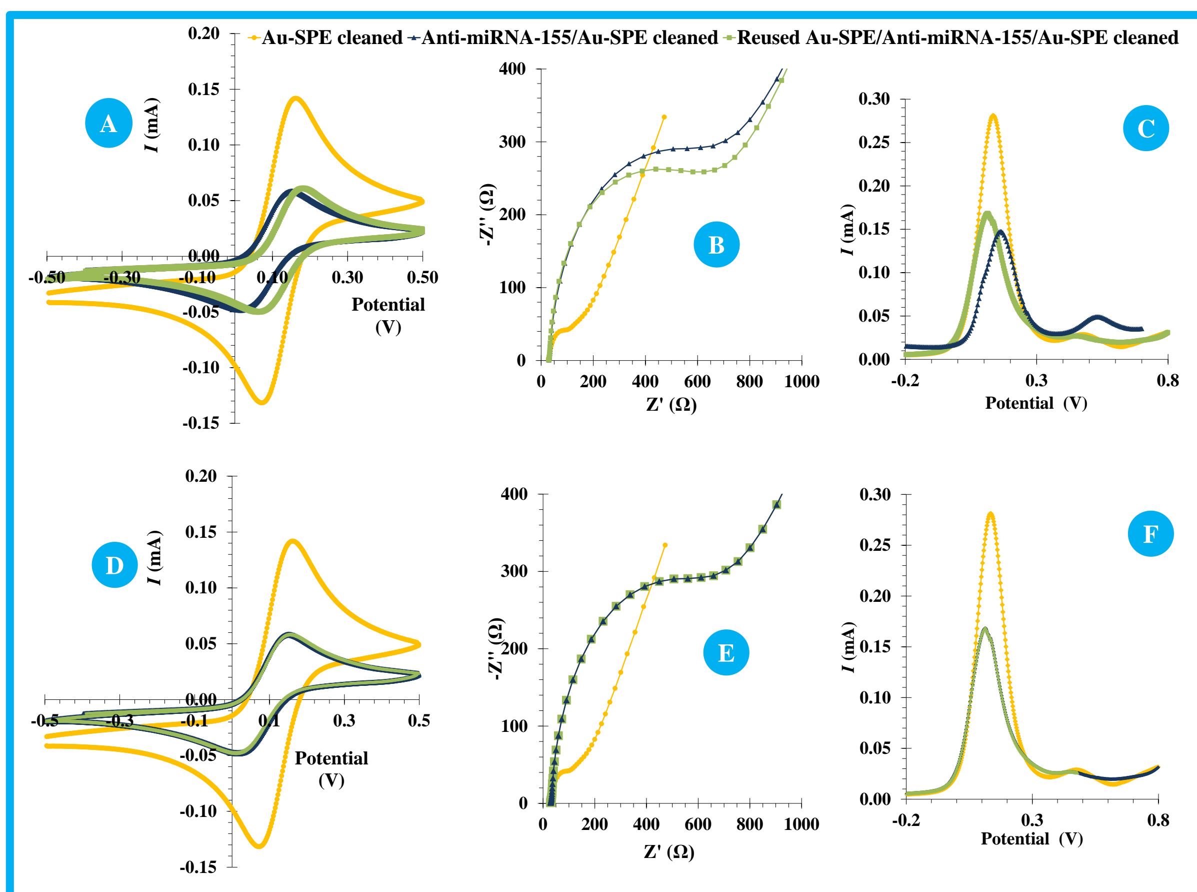


**Figure 11:** Regeneration of the biosensor (in green), at 90ºC (A, B, C) and 60 ºC (D, E, F), controlled by CV (A, D), EIS (B, E) and SWV (C, D), tested in $5.0\times10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M $[Fe(CN)_6]^{4-}$ redox probe.

After 90ºC for 5 minutes, only little alterations were detected compared to the original signal of the biosensor before its hybridization: little shift in CV peaks; (Figure 11A); little decrease of the EIS resistance (Figure 11B); and a little increase of the peak current in SWV. Still, the small changes observed in the reused Au-SPE/anti-miRNA155 biosensor seemed to be related to the silver reference electrode modification/oxidation, promoted by such a high temperature (Figure 11C). This was visually perceptible.

Thus, further assays were tested at lower temperature, in conjunction with a chemical buffer. For this, the hybridized biosensor was incubated in a saline-sodium citrate buffer (SSC) diluted 10× at 60ºC. After this, the signal of the regenerated surface matched exactly that of the original biosensor, in all electrochemical techniques (Figure 11, D, E and F).

After recovering the original signal, the reused biosensor was calibrated again to evaluate its behaviour under a second calibration, and further on in a third calibration. This was made to test the ability of the complementary oligonucleotides remaining on the surface to hybridize with its target miRNA (because staying there would not grant its ability to hybridize). The calibration assays were made by EIS and SWV measurements again, and showed very similar analytical features to the original biosensor, calibrated by its first time.

In EIS assays, the linear range was observed between 1.0 fM and 100 pM, plotting logarithm $R_{ct}$ against logarithm miRNA-155 concentration (Figure 12A). The slope was 0.0793 logΩ/decade in the second calibration and changed to 0.0786 logΩ/decade in the third calibration, while the intercept was 4.1554 logΩ in the second calibration and changed to 4.1364 logΩ in the third calibration. The standard deviation of the slope and intercept of all three calibrations performed in the same device was 5.2 and 0.3 %, meaning that the biosensor is able to generate reproducible calibrations after regeneration. Indeed, it is possible that the regeneration process has a little (but not relevant) impact on the biosensor response, as the standard deviation between second and third calibrations is much smaller (equal to 0.6 and 0.3 %, respectively).

In SWV assays the calibration was also recovered in terms of concentration range, but the variability within the three calibrations was higher. The slope of the second calibration was -26.387 µA/decade and changed to -31.929 µA/decade in the following calibration, while the intercept was 201.5 µA and changed to 257.6 µA. The standard deviation of the slope and intercept of the three calibrations (first and two regenerated) was 9.5 and 16.0 %, which was clearly high, when compared with the EIS assays.

Overall, the biosensor may be regenerated after contacting with miRNA concentrations up to 100 nM, provided that the suitable approach is taken. In addition, EIS studies seem more reliable, if the Au-SPE is to be reused after its first calibration. This is particularly suitable for making the complete analytical procedure with a single chip (which may include calibration and sample analysis, all in a single device).

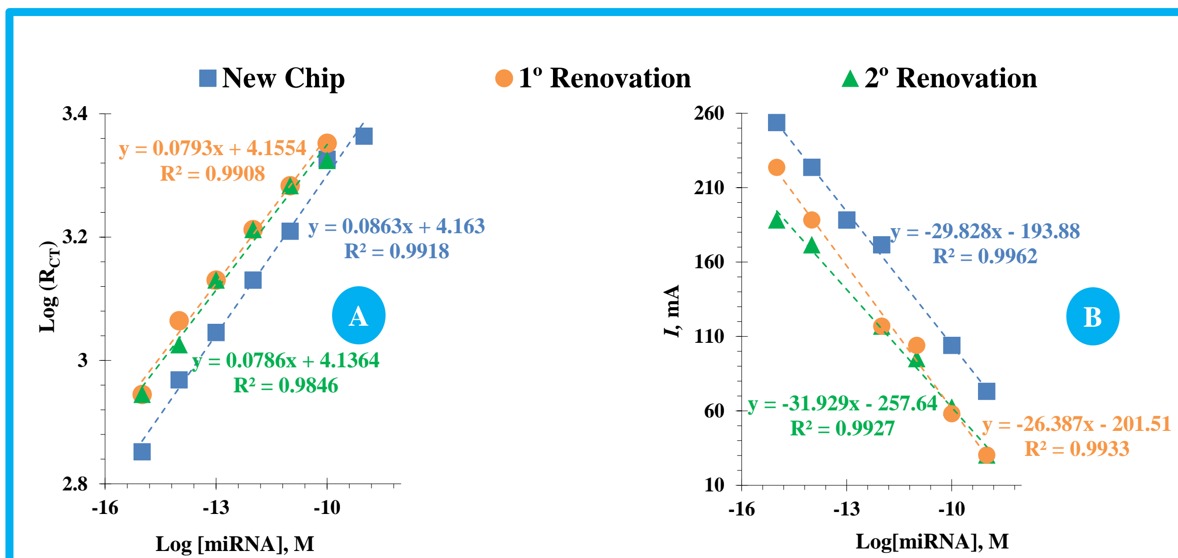


**Figure 12:** Consecutive calibrations in the same chip, first and after regeneration, (A) in EIS and (B) in SWV assays, performed in $5.0\times10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M $[Fe(CN)6]^{4-}$, in buffer (TRIS and EDTA with NaCl), pH 7.4, in the same concentration range of miRNA-155 as before.

### *III.3.4.7. Selectivity*

The selectivity study was conducted by evaluating the effect of chemical compounds present in biological fluids. A competitive assay was selected for this purpose. This was done by testing a solution of 10 pM miRNA-155 as single analyte and also in conjunction with other common biomolecules in serum, keeping the same concentration of miRNA-155. The incubation time was set to 30 minutes, the same period of time used in the calibration.

The interfering compounds studied were CA-15-3 (protein biomarker that in breast cancer condition co-exists with miRNA-155 in serum) and BSA (highly concentrated protein in serum of normal/diseased person). These species were tested within their physiological levels. The % average deviation produced by each interfering species in pure miRNA-155 solutions was 3.8 % when CA-15.3 was present and 5.17 % when BSA was added (Figure 13). These assays were performed in triplicate using the same Au-SPE, accounting its reliability after regeneration in EIS measurements.

The extract of cell lines from melanoma was also tested herein. Melanoma is another condition of cancer where miRNA-155 is not expected to exist, at least in significant levels. The obtained signal was almost coincident to the blank, therefore indicating that no significant interference would be expected by applying this biosensor to real conditions. Overall, no significant interferences were observed in these tests, neither with single analytes nor with complex real cell extracts.

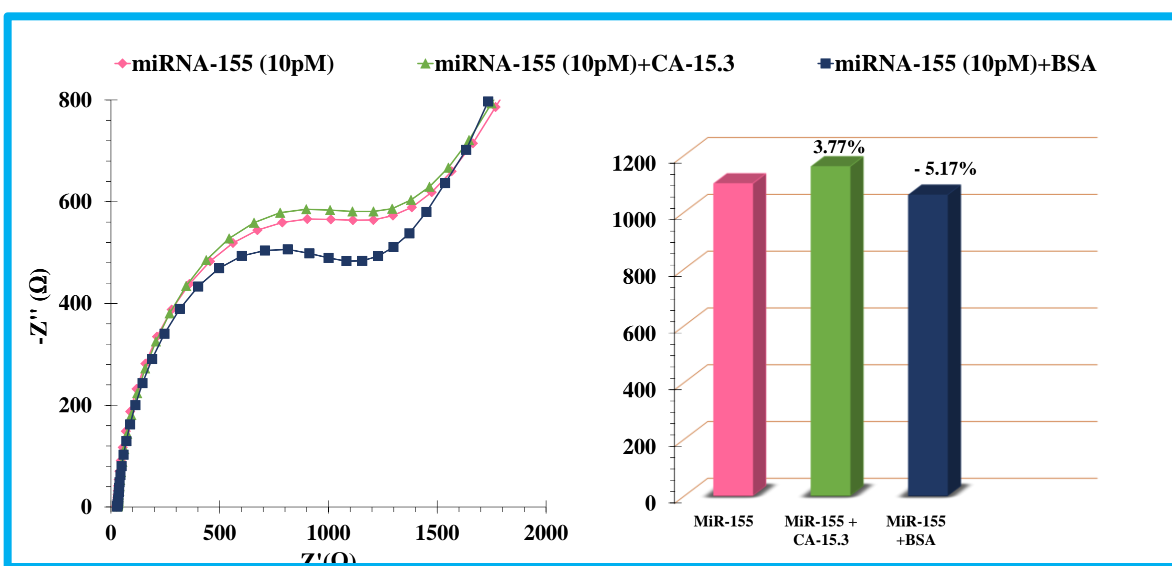


**Figure 13:** Selectivity behaviour of the biosensor for miRNA-155 (10 pM) against CA-15.3 (30 U/mL) and BSA (0.30 µg/mL), after 30 minutes incubation and for the same redox probe tested before.

### *III.3.4.8. miRNA-155 assay in human serum samples*

The application of the biosensor to real samples was made after calibrating in standard solutions prepared in blank serum, instead of the buffer used until now. The analytical features of the resulting calibration curves in EIS and SWV were also evaluated at this stage.

As before, EIS calibration curves plotted logarithm $R_{ct}$ (in EIS) against logarithm miRNA-155 concentration. In general, the calibrations showed good analytical features in terms of lower concentration of linear range, LOD and slope (10aM, 5.7aM, 0.082 logΩ/decade) respectively (Figure 14B). Compared to the calibrations made with standard solutions prepared in buffer, this novel condition of preparing standards in serum had no significant impact in the slope (within the previous standard deviation), but unexpectedly had a favourable impact upon the concentration range enabling a response. The biosensor provided linear responses down to 10 aM with lower LODs than before. The calibrations in SWV

showed a similar behaviour (Figure 14C), with a decrease in the lower limit of linear range and LOD and a slope increase (1 aM, 0.18 aM, -36μA/decade, respectively).

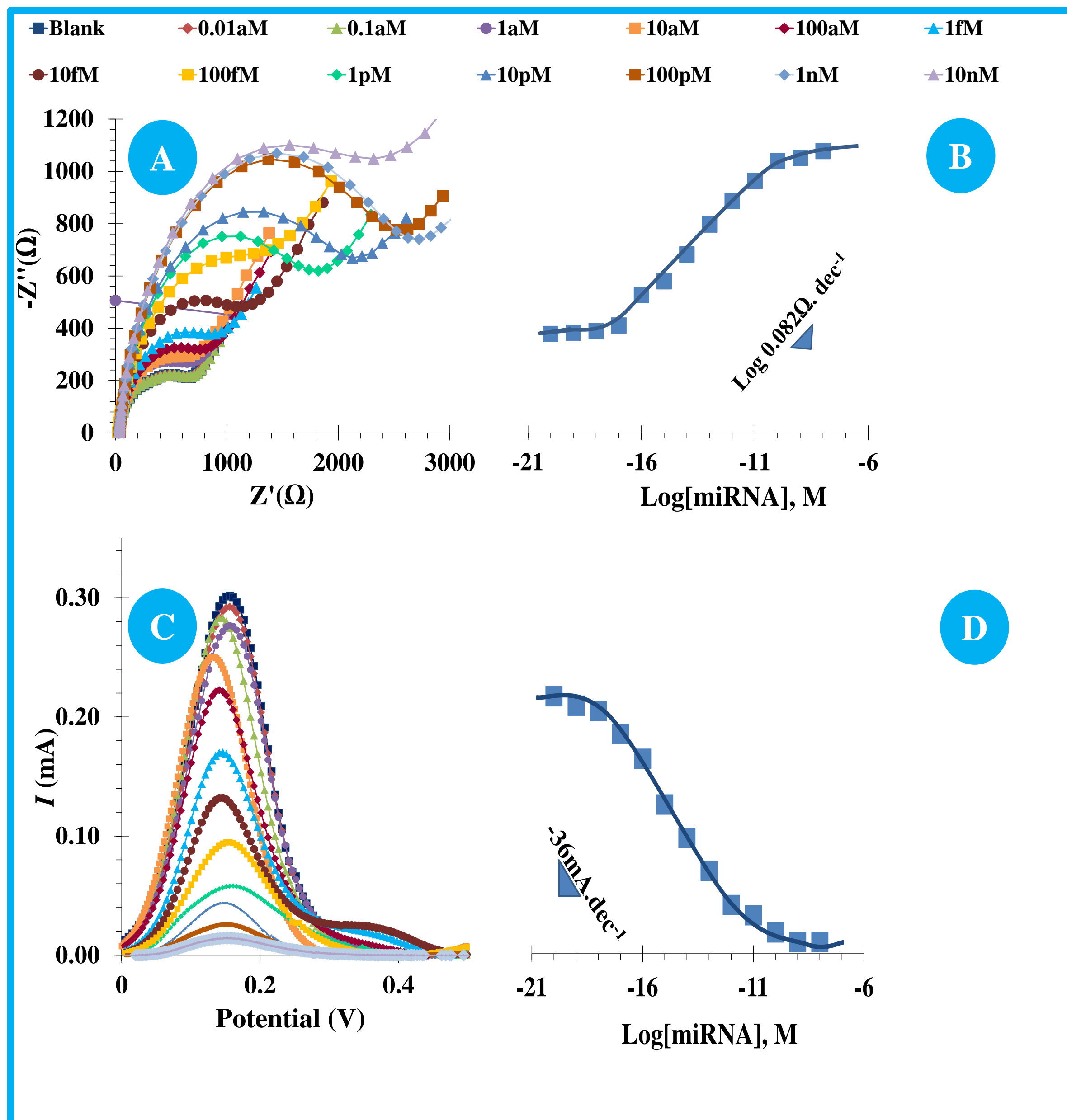


**Figure 14:** EIS(A) and SWV(C) measurements, and the corresponding calibration curves (B e D), in $5.0\times10^{-3}$ M $[Fe(CN)_6]^{3-}$ and $5.0\times10^{-3}$ M $[Fe(CN)_6]^{4-}$, in standard solutions prepared in a background of blank human serum, pH 7.4, with different concentration of miRNA-155.

For sample analysis, real human serum serving as blank was spiked with miRNA-155 in two different concentrations: 100 fM and 100 pM. This was performed in triplicate and only by following EIS data. The calibration data used in the analysis to calculate the concentrations in the spiked serum was the one provided by the same biosensor before regeneration. Comparing added and found amounts, the relative errors obtained were 6.9 and 7.2%,

respectively. Overall, the results were found accurate in close-to-real conditions, as the device was tested in a background of control human serum of healthy individual.

### III.3.5. Conclusions

This work described the development of a highly specific and very sensitive biosensor for the rapid detection of a cancer biomarker, miRNA155 present in serum. The biosensor assembly was very simple and effective, being temperature a critical step. Form the analytical perspective, the biosensor was able to detect very low concentrations of miRNA-155, down to 1-10 aM in a serum background, and could be reused for consecutive readings of new solutions, most especially in EIS. In addition, the biosensor showed excellent selectivity towards other proteins in biological fluids and cell extracts from a side-cancer condition.

This simple and sensitive strategy is considered a promising approach for the simultaneous quantitative analysis of multiple miRNA-155 in physiological fluids, in biomedical research and POC diagnosis. In addition, this device opens new horizons into real time monitoring of miRNA cancer biomarker in clinical context, also taking advantage of its ability de-hybridize and allowing reusing easily an on-line sensor.

### III.3.6. Acknowledgements

The authors acknowledge the financial support of European Research Council though the Starting Grant, ERC-StG-3P’s/2012, GA 311086 (to MGF Sales).

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# IV.CHAPTER

## IV.1. Conclusion

The miRNA can be new potential biomarkers is essential, in a way that combining various methodologies will allow having a more preventing way to confirm breast cancer and thus, apply a better treatment to the patient.

So the biosensors are a great alternative method for detect the miRNA. In this case this biosensor is simply and rapidly method and shows a good analytical performance in synthetic serum with linear range, excellent specificity and reproducibility in all tests that used.

The future work may include testing real samples, and use the mismatch method as another technique to confirm that the sensor is sensitive and specific. Also, a good alternative may be to continue the work with cell lines and compare the biosensor response with another method, such as PCR.

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