Assessing the enhancement performance of a novel aptasensor for osteopontin detection

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Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less. ― Marie Curie

Abstract

Cancer diseases are associated with the presence of a wide range of protein biomarkers. Aptasensor arrays may enable early multiple-detection of these biomarkers which can make important improvements in the lives of cancer patients. Clinical researches suggest that osteopontin, an overexpressed protein by tumor cells may be used as a diagnostic biomarker for several types of cancer. In this perspective, the objective of this dissertation was to evaluate the performance of a previously selected aptamer (C10K7) to detect osteopontin using cyclic voltammetry. The electrochemical detection of osteopontin was assessed using different screen-printed gold electrodes with the selected aptamer immobilized, being determined the detection and quantification limits. The specificity of the DNA aptasensor to other proteins was also studied using thrombin, lysozyme and bovine serum albumin which were evaluated by a previous research as possible interferents. The preliminary work carried out showed that this new aptamer allowed achieving a detection limit of 1.3 nM and a quantification limit of 4.0 nM for osteopontin. Considering that the reported range of plasma osteopontin concentrations in patients with either metastatic or recurrent breast cancer is up to 290 ng/mL (~ 4.46 nM) which is higher than the detection and quantification limits found and which suggest that the proposed aptasensor could be applied in both osteopontin detection and quantification. On the other hand, with the studied aptamer, the detection and quantification limits were lower than those reported in the literature for cyclic voltammetry, which were of 2.6 nM for a DNA aptamer (designated as C10K2) and 3.7 nM for an RNA aptamer (called OPN-R3). The repeatability assays showed a coefficient of variation equal to 7% which demonstrate the closeness of the agreement between the results. The evaluation of the specificity of the DNA aptasensor towards osteopontin showed little or almost no interference to other proteins. However, the interference study showed that the C10K7 aptamer was more prone to interferences from lysozyme and bovine serum albumin than the C10K2 aptamer, previously studied by the research team but showed a lower interference from thrombin. These results highlight the promising capability of the new DNA aptamer (C10K7) to be used in the development of an electrochemical aptasensor for the osteopontin detection, which could be foreseen as a diagnosis and therapy monitoring tool. Nevertheless, these preliminary satisfactory results need to be further checked, namely by the evaluation of its performance for the detection of human osteopontin in biological fluids like blood or plasma.

Resumo

O cancro é uma doença à qual se pode associar a presença de diversas proteínas nos fluidos biológicos de pacientes com essa patologia, as quais podem ser usadas como biomarcadores. Aptasensores baseados em diferentes aptâmeros selecionados para biomarcadores específicos podem permitir a sua deteção num estágio precoce da doença, contribuindo para melhorar a vida dos pacientes com cancro. Estudos clínicos sugerem que a osteopontina, é uma proteína sobre-expressa por células tumorais, podendo ser utilizada como biomarcador no diagnóstico de vários tipos de cancro. O objetivo do presente trabalho foi avaliar o desempenho de um aptâmero previamente selecionado (C10K7) para detectar osteopontina recorrendo à técnica de voltametria cíclica. O estudo realizado permitiu verificar que este novo aptasensor possui um limite de deteção de 1.3 nM e um limite de quantificação de 4.0 nM. Estes valores permitem antever a aplicabilidade prática deste dispositivo como uma ferramenta de deteção de osteopontina uma vez que as concentrações reportadas em plasma de pacientes com o cancro são da ordem dos 4.46 nM (290 ng/mL). Por outro lado, convém referir que o novo aptasensor apresenta limites de detecção e de quantificação inferiores aos descritos na literatura para outros aptasensores baseados em aptâmeros de RNA (OPN-R3) e DNA (C10K2), nomeadamente 3.7 nM e 2.6 nM, também estabelecidos por voltametria cíclica. Os ensaios de repetibilidade realizados mostraram que o aptasensor desenvolvido apresentava um desempenho satisfatório (coeficientes de variação inferiores a 7%). Por fim, o novo aptasensor mostrou-se bastante específico relativamente à osteopontina, molécula alvo, com reduzida interferência por parte de outras protéinas tipo (lisozima, albumina bovina e trombina). Os resultados obtidos sãp bastante promissores indicando o novo aptasensor de DNA (C10K7) uma possível ferramenta de diagnóstico e/ou monitorização da evolução do cancro. No entanto, o desempenho satisfatório descrito terá de ser validado na deteção de osteopontina humano em líquidos biológicos como o sangue ou o plasma.

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ΔEp - Potential variation between cathodic and anodic peaks

ΔG - Gibbs free energy

ΔI - Relative current change

Au - Gold

BSA - Bovine serum albumin

BSP - Bone sialoprotein

BTA - Bladder tumour antigen

CA-125 - Carbohydrate antigen-125

CEA - Carcinoembryonic antigen

CV - Cyclic voltammetry

DEPC - Diethypyrocarbonate

DMP1 - Dentin matrix protein 1

DNA - Deoxyribonucleic acid

DPA - 3,3-dithiodipropionic acid

DPV - Differential pulse voltammetry

DSPP - Dentin sialophosphoprotein

ELISA - Enzyme-linked immunosorbent assays

ER - Estrogen receptor

ETA - Ethanolamine

FDA - Food and Drug Administration

HAS - Human serum albumin

HE4 - Human epididymis protein 4

HER2 - Human epidermal growth factor receptor 2

HIF-1α - Hypoxia-inducible factor 1.

Ip - Peak current intensity

Ipa - Anodic peak current

Ipc - Cathodic peak current

IUPAC - International Union of Pure and Applied Chemistry

$K_3Fe(CN)_6$ - Potassium hexacyanoferrate (III)

$K_4Fe(CN)_6$·- Potassium hexacyanoferrate (II)

KCl - Potassium chloride

KH_2PO_4 - Potassium dihydrogen phosphate

LOD - Detection limit

LOQ - Quantification limit

LYS - Lysozyme

MEPE - Matrix extracellular phosphoglycoprotein

MMP - Metalloproteinase

NaCl - Sodium chloride

NHS - N-hydroxysuccinimide

NSCLC - Non-small-cell lung carcinoma

NSE - Neuron-specific enolase

OPN - Osteopontin

PBS - Phosphate buffer saline

PI3K - Phosphatidylinositol-3 kinase

PR - Progesterone receptor

Pro2PSA1 - Pro-prostate specific antigen

rhOPN - Recombinant human OPN

RNA - Ribonucleic acid

ROMA - Risk of ovarian malignancy algorithm

SELEX - Systematic Evolution of Ligands by Exponential Enrichment

SPGEs - Screen-printed gold electrodes

SWV - Square wave voltammetry

THR - Thrombin

TPS - Tissue polypeptide specific antigen

VEGF - Vascular endothelial growth factor

WE - Working electrode

1. Introduction

1.1. Cancer

The general term "cancer" refers to a broad group of diseases that can affect any part of the body (Bhatt et al., 2010). The apparition of cancer is due to the transformation of normal cells into tumour cells, a process that goes through many stages, with a classic progression to a precancerous lesion and after that to a malignant tumour (Bhatt et al., 2010). These modifications come from interactions between external agents and the genetic factors specific to the subject (Bhatt et al., 2010). 18.1 million new cases and 9.6 million cancer deaths were estimated to occur worldwide in 2018 (Bray et al., 2018). Table 1 presents the new cases and deaths for the top 10 cancers in 2018. It is estimated that nearly one-half of the cases and over one-half of the cancer deaths in the world will occur in Asia in the year 2018, especially because close to 60% of the global population resides there (Bray et al., 2018). Europe accounts for 23.4% of the total cancer cases worldwide and 20.3% of the cancer deaths, even though it represents only 9% of the global population, followed by the Americas' 21% of incidence and 14.4% of mortality worldwide (Bray et al., 2018).

For both sexes combined, lung cancer is the most commonly diagnosed cancer with 11.6% of the total cases and the leading cause of cancer death with 18.4% of the total cancer deaths, then followed closely by female breast cancer with 11.6%, colorectal cancer with 10.2%, and prostate cancer with 7.1% (Bray et al., 2018). By sexes, lung cancer is the most frequently diagnosed cancer and the leading cause of cancer death in males, followed by colorectal and prostate cancer for incidence, and stomach and liver cancer for mortality. Among females, breast cancer is considered the most commonly diagnosed cancer and the leading cause of cancer death, followed by colorectal and lung cancer (Bray et al., 2018).

Table 1. New cases and deaths for the top 10 cancers in 2018.

Cancer site	Number of new cases	Number of deaths
Lung	2,093,876	1,761,007
Breast	2,088,849	626,679
Prostate	1,276,106	358,989
Colon	1,096,601	551,269
Nonmelanoma of skin	1,042,056	65,155
Stomach	1,033,701	782,685
Liver	841,080	781,631
Rectum	704,376	310,394
Esophagus	572,034	508,585
Cervix uteri	569,847	311,365

One of the hallmarks of cancer is the rapid proliferation of abnormal cells that, beyond their usual boundaries, can invade adjacent parts of the body and then spread to other organs. This is known as metastasis, which is the leading cause of death from cancer. This proliferative potential and survival advantage of cancer cells are caused by alterations in the status and expression of primarily three main classes of genes:

Proto-oncogenes: Proto-oncogenes are a group of genes that lead normal cells to become cancerous when they are mutated (Adamson et al.,1987; Weinstein et al., 2006).

Mutations in proto-oncogenes are in general dominant in nature, and we call the mutated version of a proto-oncogene an oncogene. Frequently, proto-oncogenes encode proteins that function to stimulate cell division, inhibit cell differentiation, and halt cell death. All of these processes are important for normal human development and for the maintenance of organs and tissues. Oncogenes, nonetheless, typically exhibit increased production of these proteins, therefore leading to increased cell division, decreased cell differentiation, and inhibition of cell

death, if we take them together, we can say that these phenotypes define cancer cells. Thus, oncogenes are currently a major molecular target for anti-cancer drug design (Weinstein et al., 2006).

Tumour suppressor genes: A tumour suppressor gene, or antioncogene, is a gene that protects a cell from one step on the path to cancer (Weinberg et al., 2014). When this gene mutates to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes. The loss of these genes may be even more important than proto-oncogene/oncogene activation for the formation of many kinds of human cancer cells. tumour suppressor genes can be grouped into categories including caretaker genes, gatekeeper genes, and landscaper genes, the classification schemes are evolving as medicine advances, learning from fields including molecular biology, genetics, and epigenetics (Weinberg et al., 2014).

DNA repair genes: DNA repair is a collection of processes by which a cell identifies and corrects damage to the DNA molecules that encode its genome (Wood et al., 2001). In human cells, both abnormal metabolic activities and environmental factors such as radiation can cause DNA damage, resulting in as many as 1 million individual molecular lesions per cell per day. Many of these lesions cause structural damage to the DNA molecule and can alter or eliminate the cell's ability to transcribe the gene that the affected DNA encodes. Other lesions induce potentially harmful mutations in the cell's genome, which affect the survival of its daughter cells after it undergoes mitosis. As a consequence, the DNA repair process is constantly active as it responds to damage in the DNA structure. When normal repair processes fail, and when cellular apoptosis does not occur, irreparable DNA damage may occur, including double-strand breaks and DNA cross-linkages (interstrand crosslinks or ICLs). This can eventually lead to malignant tumours or cancer. The accumulation of errors can overwhelm the cell and result in cancer (Wood et al., 2001) and when faulty genes are inherited from parents, the risk of cancer is higher (Harris, 2015). On the other hand, the detection of advanced cancer and the inability to access diagnosis and treatment are common problems.

Finally, treatment is more likely to be effective, with a better chance of survival, reduced morbidity and lower the costs if the cancer is diagnosed in an early stage. Early diagnosis is useful in all situations for the majority of cancers. When the diagnosis is made at an advanced stage, curative treatment is sometimes no longer possible, that’s why researchers are trying to develop new tools and methods that can help detect cancer in an early stage, in this context it is possible to talk about biomarkers, which are attracting more and more scientists who suggest

that it is possible to detect cancer at an early stage thanks to their specificity (Meirinho et al., 2015).

1.2. Cancer biomarkers

Cancer biomarkers are the substances produced by normal cells as well as cancer cells in response to cancer that can be found in the blood, tumour tissue, urine or other body fluids (Gam., 2012). National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".

Biomarkers can be used for patient assessment in many clinical settings, including estimating risk of disease, screening for occult primary cancers, determining prognosis and prediction for patients diagnosed with cancer, distinguishing benign from malignant findings or one type of malignancy from another, and monitoring status of the disease, either to detect recurrence or determine progression or response to therapy (Henry et al., 2012).

For example, the Pro2PSA1 which is a biomarker for prostate cancer, it is used in discriminating cancer from benign disease. Also, the OVA1 (multiple proteins) which is a biomarker for ovarian cancer, it's used in the prediction of malignancy, we have as well the CA27.29, a biomarker of breast cancer, its clinical use is monitoring disease response to therapy (Wei et al., 2016).

There are three main categories of cancer biomarkers, namely diagnostic, prognostic and predictive biomarkers (Goossens et al., 2015). A diagnostic biomarker is used to detect the presence of the disease and is present in any stage during cancer development (Mishra et al., 2010). Bladder tumour antigen (BTA) and nuclear matrix protein-22 (NMP-22) are examples of approved diagnostic biomarkers by the Food and Drug Administration (FDA) for bladder cancers (Lau et al., 2009). A prognostic biomarker is used to predict the course of the disease and to indicate the aggressiveness of the tumour (Gam, 2012). As an example, the glycoprotein CA125 is a prognostic biomarker of ovarian cancer (Huang et al., 2010). A predictive marker is used to predict the response of a patient to particular therapeutic interventions. For instance, the predictive biomarker human epidermal growth factor receptor HER2 can tell us about the response to trastuzumab in breast cancer (Henry et al., 2012).

On the basis of biochemical molecules, cancer biomarkers have a broad range of entities, like RNA, DNA, micro RNA, proteins, lipids, carbohydrate and small metabolites, cytokinetic and

cytogenetic parameters found in the body fluid (Bhatt et al., 2010). Proteomic markers are the most important biomarkers since they are the main executioner molecules in cells, they are also more relevant to the disease state initiation and progression (Srivastava et al., 2005).

Unfortunately, most of the available biomarkers lack the specificity and sensitivity to be used in early detection (Gam, 2012). For example, CA27.29, CA15.3, and CEA are breast cancer biomarkers approved by the FDA but they are useful only for monitoring advanced breast cancers (Mirza et al., 2008).

1.2.1. Examples of cancer biomarkers

ROMA (HE4+CA-125): Ovarian Cancer biomarkers:

Ovarian cancer is one of the three most common malignant tumours in the female reproductive system. It has an insidious onset with a difficult early diagnosis (Wei et al., 2016). In approximately 70% of all cases of ovarian cancer, the disease is not diagnosed before reaching an advanced stage (Zhang et al., 2004). The 5-year survival rate associated with ovarian cancer is lower than 30% (Heintz et al., 2006). Over 90% of all cases of ovarian masses detected in premenopausal and 60% in postmenopausal women are benign (Enakpene et al., 2009). The early diagnosis of ovarian malignant tumour becomes a key factor in improving the survival rate of patients. Tools currently in use for differentiating between low- and high-risk patients with ovarian cancer are the tumour markers carbohydrate antigen-125 (CA-125) and the human epididymis protein 4 (HE4), as well as the value of the risk of ovarian malignancy algorithm (ROMA) (Karlsen et al., 2012). Table 2 presents the diagnostic values of CA-125, HE4 and ROMA in ovarian cancer compared with the standard.

Table 2. The diagnostic values of CA-125, HE4 and ROMA in ovarian cancer and benign tumour compared with the healthy control (Wei et al., 2016).

Parameters	Healthy Control group	Benign tumour group	Ovarian cancer group
Cases	30	64	64
HE4 (pmol/l)	39.04 ±8.38	54.76 ±42.35	739.04 ±860.04
CA-125 (U/ml)	15.08 ±5.28	49.07 ±175.61	868.85 ±1204.08
ROMA index	6.18 ±2.21	10.15 ±11.98	76.30 ±28.57

ROMA index = $8.09 + 1.04 \times \ln(\text{HE4}) + 0.732 \times \ln(\text{CA-125})$

CEA, NSE, TPS: Lung cancer biomarkers:

Carcinoembryonic antigen (**CEA**) describes a set of highly related glycoproteins involved in cell adhesion. CEA is normally produced in gastrointestinal tissue during fetal development, but the production stops before birth. Consequently, CEA is usually present at very low levels in the blood of healthy adults (about 20 ng/mL). However, the serum levels are raised in some types of cancer, which means that it can be used as a tumour marker in clinical tests. Serum levels can also be elevated in heavy smokers (Asadet al., 2016).

Neuron-specific enolase (NSE) is an important indicator for monitoring tumour progression in the lung cancer patient. Previous studies suggested that the higher the NSE, the worse prognosis of NSCLC patients (Stosic, 2006).

Tissue polypeptide specific antigen (TPS) is valuable to diagnosis, response monitoring for patients with lung cancer, moreover, it may be a useful factor of prognosis of non-small cell lung cancer (Wang et al., 2010). Additionaly, some biomarkers are more specific to a certain type of cancers than others. Table 3 presents the comparative levels of CEA, NSE and TPS in the blood plasma of patients with adenocarcinoma and squamous lung cancer and healthy people.

Table 3. Comparative levels of well-known lung cancer biomarkers (CEA, NSE, TPS) in the blood plasma of patients with adenocarcinoma and squamous lung cancer and healthy people (Stosic, 2006).

Tumor-Associated Protein	Adenocarcinoma	Squamous Carcinoma	Normal
CEA	30.76 ng/mL	4.49 ng/mL	<5.0 ng/mL
NSE	17.95 ng/mL	16.83 ng/mL	15.7–17.1 ng/mL
TPS	10–3842 ng/mL	0–3000 ng/mL	34.9 ng/mL

A list of FDA-approved protein tumour markers used in clinical practice and mentioned previously is shown in Table 4.

Table 4. List of FDA-approved protein tumour markers currently used in clinical practice (adapted from Ry et al., 2013).

Biomarker	**Clinical use**	**Cancer type**	**Specimen**
Pro2PSA	Discriminating cancer from benign disease	Prostate	Serum
ROMA (HE4+CA-125)	Prediction of malignancy	Ovarian	Serum
OVA1 (multiple proteins)	Prediction of malignancy	Ovarian	Serum
HE42	Monitoring recurrence or progression of the disease	Ovarian	Serum
Fibrin/fibrinogen degradation product (DR-70)	Monitoring progression of the disease	Colorectal	Serum
AFP-L3%	Risk assessment for the development of disease	Hepatocellular	Serum
p63 protein	Aid in differential diagnosis	Prostate	FFPE tissue
Circulating tumour Cells (EpCAM, CD45, cytokeratins 8, 18+, 19+)	Prediction of cancer progression and survival	Breast	Whole blood
c-Kit	Detection of tumours, aid in the selection of patients	Gastrointestinal tumours	FFPE tissue
CA319-9	Monitoring disease status	Pancreatic	Serum, Plasma
Estrogen receptor (ER)	Prognosis, response to therapy	Breast	FFPE tissue
Progesterone receptor (PR)	Prognosis, response to therapy	Breast	FFPE tissue
HER-2/neu	Assessment for therapy	Breast	FFPE tissue
CA-125	Monitoring disease progression, response to therapy	Ovarian	Serum, plasma
CA15-3	Monitoring disease response to therapy	Breast	Serum, plasma

Table 4. (continued).

CA27.29	Monitoring disease response to therapy	Breast	Serum
Free PSA4	Discriminating cancer from benign disease	Prostate	Serum
Thyroglobulin	Aid in monitoring	Thyroid	Serum, plasma

Note: Pro2PSA (pro-prostate specific antigen); HE4 (Human epidymis protein); CA19-9 (caner antigen); PSA (Prostate-specific antigen).

Actually, several works reported osteopontin as an important candidate biomarker for human cancer (Psyrri et al., 2017; Hao et al., 2016; Ferreira et al., 2016; Nassar et al., 2015; Weber., 2011; Ahmed et al. 2011). But it has not yet been used in clinical diagnostics.

1.3. Osteopontin

1.3.1. Definition of osteopontin

Osteopontin (OPN) is a negatively charged aspartic acid-rich, N-linked glycosylated phosphoprotein and it is composed of 314 amino acid residues (Kadkol et al., 2006; Scatena et al., 2006). Figure 1 presents the tertiary structure of the osteopontin. The localization of the human gene for osteopontin is in the long arm of chromosome 4q13 directly related to four similar genes encoding for bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), and matrix extracellular phosphoglycoprotein (MEPE) (Fedarko et al., 2004). Due to common functional motifs and domains, these five integrin-binding glycophosphoproteins are categorized as the so-called SIBLING proteins (small integrin-binding ligand N-linked glycoproteins) (Bellahcene et al., 2008). Osteopontin is encoded by a single copy gene, but as a result of alternative splicing, alternative translation and different post-translational modifications (PTMs), it exists in various isoforms, which allow for a molecular weight ranging from 41 to 75 kDa (Anborgh et al., 2011). OPN has primarily been described as a secreted protein involved in several physiological as well as pathological events. However, current evidence suggests that OPN can also be found in the cytoplasm and the nucleus (Junaid et al., 2007). This form of intracellular OPN (iOPN) is the result of alternative translation and has biological functions distinct from those of secreted OPN (sOPN) (Inoue et al., 2011).

OPN contains an integrin-binding RGD sequence which interacts with CD44v 6/7, that is a cell surface glycoprotein involved in cell-cell interactions. OPN can also activate intracellular pathways to regulate gene expression within the immune system (Zhao et al., 2018).

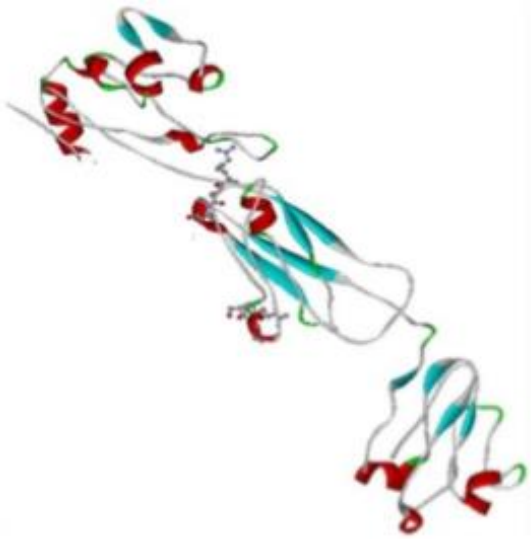

Figure 1. Tertiary structure of the osteopontin (Sivakumar et al., 2014).

Clinical studies have revealed that higher expression of osteopontin is found in tumour tissue and serum of different types of cancers suggesting that osteopontin may be used as a diagnostic and prognostic biomarker for various cancers (Weber et al., 2010). Actually, altered osteopontin levels have been associated with breast cancer (Psyrri et al., 2017; Macrì et al., 2009), thyroid cancer (Ferreira et al. 2016), leukemia (Liersch et al., 2015), colorectal cancer (Huang et al., 2016), lung cancer (Shojaei et al., 2012), ovarian cancer (Huang et al., 2010), gastrointestinal stromal tumours (Hsu et al., 2010), pancreatic (Collins et al., 2012) and renal cell carcinoma (Matušan-Ilijaš et al., 2011). Osteopontin acts like an important molecule that is involved in all the stages of cancer progression including tumour invasion, angiogenesis and metastasis (Ahmed et al., 2011). There is well-documented evidence to suggest that osteopontin contributes to tumour progression (Zhao et al., 2018). It acts as an important and overexpressed molecule at different steps of the cancer process. Added to that, OPN has demonstrated a role in the development of various solid organ tumours via different mechanisms. As presented in figure 2 by Zhao et al. (2018), in breast, brain, prostate and ovarian cancers, OPN has been shown to preferentially bind to a variety of integrins including αvβ1, αvβ5, αvβe and αvβ3, that result in an increase in cell adhesion, migration, and invasion, whilst OPN has been shown to

bind to both integrin and CD44 receptors in lung cancers (Zhao et al., 2018). In addition to receptor binding, OPN is involved in enhancing MMP release and as a result increasing cell invasiveness and tumour growth in brain, liver, ovarian, colorectal, pancreas and prostate, and oesophageal and gastric cancers. OPN-mediated upregulation of the PI3K/Akt signaling pathway is a common feature of liver, lung, ovarian and prostate tumour progression, thus preferentially regulating cell survival, cell cycle progression and cellular growth in favor of tumour development (Zhao et al., 2018). The activation of VEGF and its downstream effector, HIF-1α, by OPN may occur dependently or independently of PI3K/Akt activation and promotes tumour angiogenesis, recruitment of endothelial cells and tumour growth, particularly in the colorectal, pancreatic, lung, and esophageal and gastric malignancies. Activation of the JNK pathway by OPN has been shown to be most specific to colorectal cancer, whilst the precise role of OPN in bladder and kidney cancers, particularly, remains to be elucidated.

Eventually, it has been demonstrated that high levels of OPN favor the survival and proliferation of cancer cells at the primary site (Zhao et al., 2018).

However, further research is necessary to improve the current understanding of these various molecular pathways and elucidate the precise role of OPN in mediating cancer progression and metastasis. Finally, OPN has the potential to be a novel biomarker and anti-cancer therapeutic target (Zhao et al., 2018) that’s why it is important to focus on OPN detection methods.

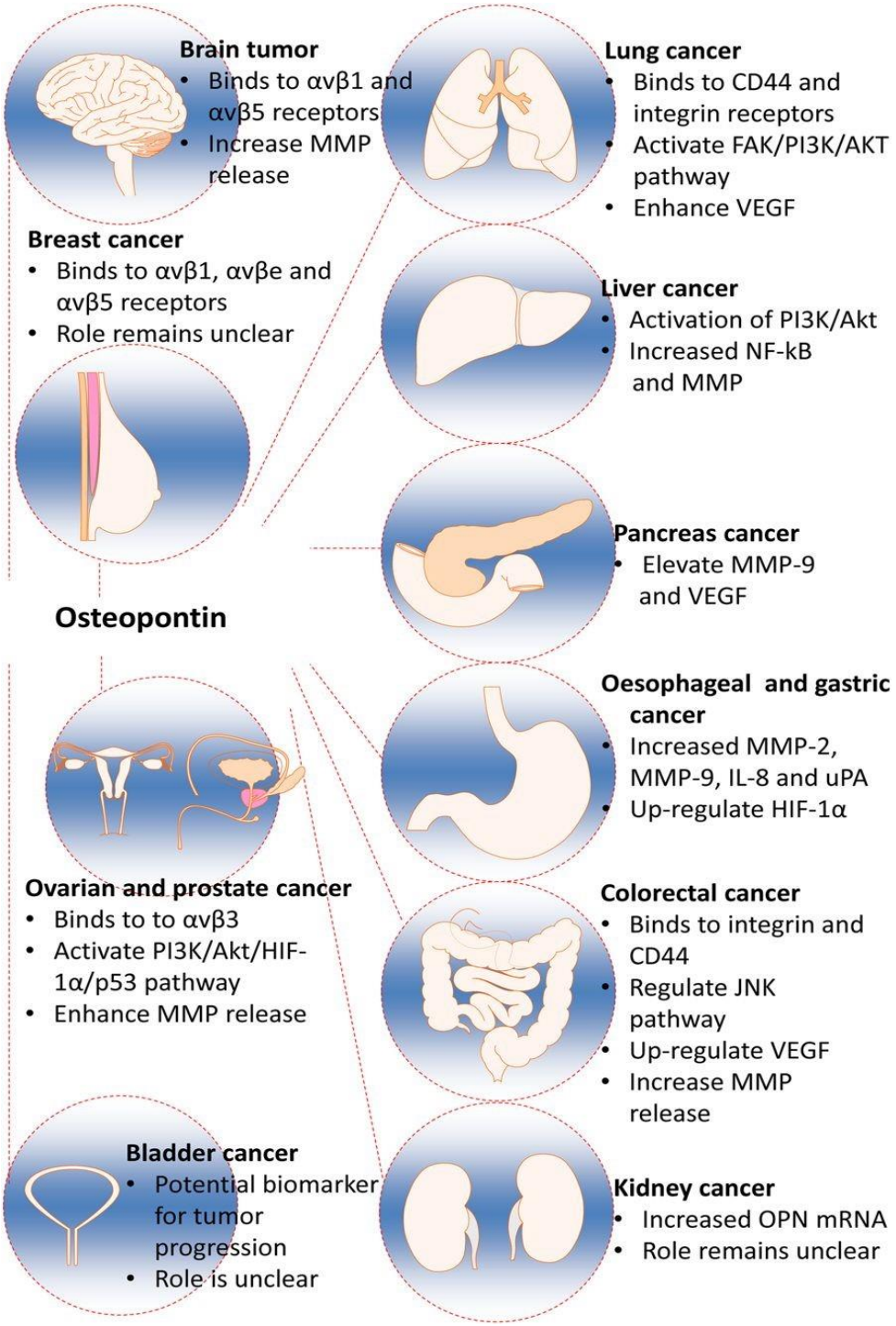

Figure 2. The role of osteopontin in various solid organ tumours (Zhao et al., 2018).

1.3.2. Osteopontin detection

The enzyme-linked immunosorbent assay (ELISA) method currently dominates the field of protein quantification and detection (Li et al., 2016). Bramwell et al. (2014) used an ELISA technique to measure the osteopontin levels in plasma samples of patients with breast cancer.

In general, ELISA is a biochemical test that uses antibodies and an enzyme-mediated color change to detect the presence of an antigen like proteins, hormones, peptides or antibody in a given sample (Gan et al., 2013). Although its widespread use, there are some limitations that have to be considered. In fact, nonspecific binding of the antibody or antigen to the plate will lead to a falsely high-positive result. In addition to that ELISA cannot distinguish between antigenically identical analytes. For now, the same ELISA assay will often recognize many or all different isoforms of the same protein in a sample. Additionally, the assay requires some specialized equipment, like a spectrophotometric microplate reader, large amounts of sample and skilled technicians (Chatziharalambous et al., 2016; Li et al., 2016). Therefore, it is urgent to develop new detection techniques for protein cancer biomarkers that circumvent the above-mentioned limitations of conventional ELISA. In that case, the biosensor is considered a promising alternative for osteopontin detection.

1.4. Biosensors

According to the International Union of Pure and Applied Chemistry (IUPAC) “a biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transducer element” (Labuda et al., 2010; Thévenot et al., 2001). Another commonly used definition for biosensors is “analytical devices that are based on a bioreceptor and that are capable of sensing biologically-relevant analytes with either electrical or optical readout” (Cheng et al., 2009).

They are powerful analytical devices that consist of a bioreceptor compound such as an antibody or nucleic acid immobilized on a transducer surface, which is capable of providing a signal due to the interaction between the bioreceptor and the analyte (Vigneshvar et al., 2016). Figure 3 presents the schematic illustration of a biosensor. Biosensors are able to detect a wide range of analytes in complex matrices and have been applied in many fields such as food industry, medical field and environmental monitoring. They provide better stability and sensitivity as compared with conventional methods such as enzyme-linked immunosorbent assays, 2-D western blotting and 2-D gel electrophoresis (Kraatz et al., 2015; Mehrotra, 2016). The main components of biosensors are:

- The bioreceptors (biorecognition elements) which could specifically recognize and identify the target (Han et al., 2010).

- Signal transducer, which could transform the biological signal into an electrical signal with high sensitivity and minimum disturbance to the measured analyte.
- A display which transforms the measured electrical/optical signal into a digital format for end-users (Cheng et al., 2009; Han et al., 2010).

The existing biosensors can be classified into immunosensors, enzymosensors, aptasensors, etc. based on the nature of the bioreceptor such as antibodies, enzymes and aptamers, respectively (Mehrotra., 2016). The principal components of all biosensors are transducers or the detector devices, displays or electronic parts comprised by a signal amplifier and the data processor, and the bioreceptors or biorecognition elements (Cheng et al., 2009; Strehlitz et al., 2008; Velusamy et al., 2010).

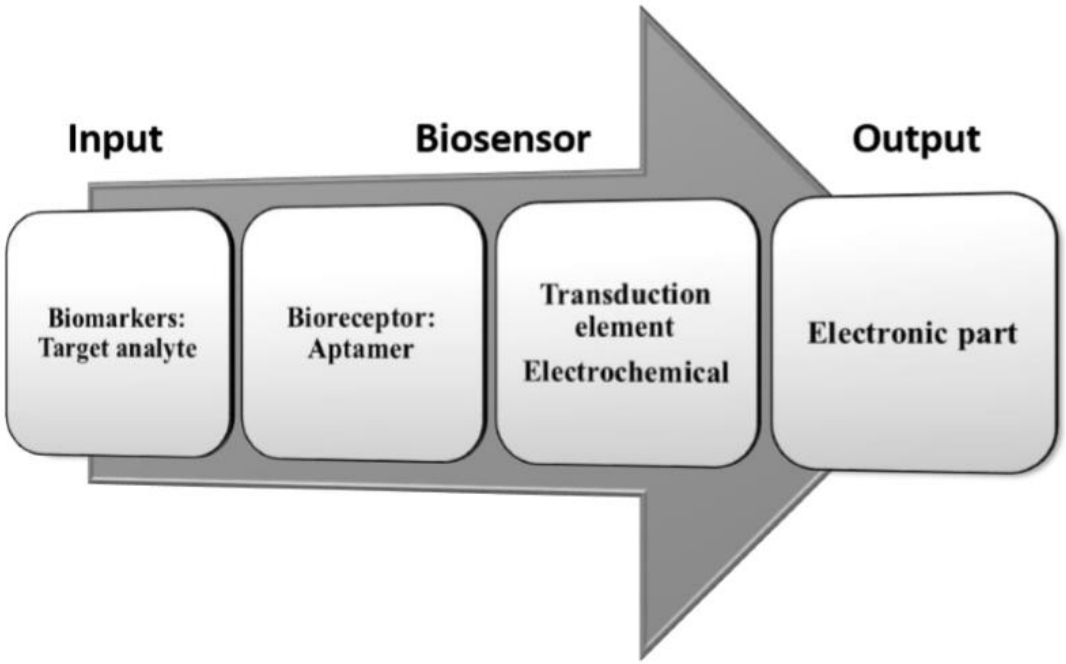

Figure 3. Schematic illustration of a biosensor (Meirinho, S.G., 2016).

The general purpose of a biosensor is to produce either discrete or continuous signals that are proportional to a property of a single target or a related group of analytes. After that, the signals are transformed into a digital format that can be recognized by the end users (Strehlitz et al., 2008). The interaction between the bioreceptor and the target is the result in one or more physicochemical modifications like electron transfer, pH modifications, mass variation, heat transfer and gases or ions release that the transducer can detect and measure them (Kumar et al., 2012). The bioreceptor will be responsible for the specific sensor response to a given target or group of targets of interest, therefore minimizing the interference from other substances in complex mixtures (Viswanathan et al., 2008). The choice of the bioreceptor is very important

in the development of a biosensor because it determines its selectivity and specificity. On the other hand, the transducer determines the sensitivity of the biosensor and is responsible for converting the biological signal into a measurable signal (Monošík et al., 2012; Sassolas et al., 2009). Usually, biosensors can be classified according to the bioreceptors type and signal transduction or transducer. Bioreceptors are generally grouped in five classes, namely enzymes, antibodies/antigens, nucleic acids, cellular structures/cells and recently biomimetic materials or synthetic bioreceptors (e.g. aptamers) (Velusamy et al., 2010). Added to that, according to the method used for the signal transduction, the biosensor can be grouped in different classes, such as electrochemical, optical and mass sensitive (Grieshaber et al., 2008). Biosensors are an attractive analytical tool in several areas due to the selectivity and sensitivity of the bioreceptor and the transducer and they are applicable to a large variety of samples including body fluids, food, cell cultures and environmental samples (Cheng et al., 2009; Grieshaber et al., 2008; Sassolas et al., 2009). The specific application of a biosensor determines the best electrochemical technique to use, as long as each detection method presents advantages and disadvantages. The electrochemical transduction presents considerable advantages over optical, piezoelectric or thermal detection (Deng et al., 2013; Radi, 2011), such as high sensitivity and selectivity, the ability to work with turbid samples, inherent miniaturization, compatibility with novel microfabrication technologies, disposability and accuracy, simplicity, robustness, possibility of usage for on-line control, fast response, and relatively low manufacturing cost, all of these advantages are making electrochemical aptasensors very attractive for diagnostic and use in point-of-care devices, also for simultaneous multi-analyte detection (Arshak et al., 2009; Radi, 2011; Saberian et al., 2011; Song et al., 2008; Velasco-Garcia and Missailidis, 2009; Velusamy et al., 2010; Xu et al., 2009).

1.5. Aptasensor: a class of biosensor

An aptasensor is a particular class of biosensor where the biological recognition element is a DNA or an RNA aptamer. In an aptasensor, the aptamer recognizes the molecular target towards which it was previously selected *in vitro* (Duan et al., 2016). Compared to other existing biological recognition elements, aptamers exhibit several advantages in terms of stability, design flexibility, and cost-effectiveness. To name a few, stability to long-term storage and under a wide range of buffer conditions, easy synthesis, resistance to denaturation and degradation, reversible thermal denaturation and ability to bind to targets with selectivity,

specificity, and affinity, equal and often superior to those of monoclonal antibodies (Lowe 2008; Meirinho et al., 2015; Moreno, 2014).

1.5.1. Electrochemical aptasensors

In electrochemical aptasensors an electrode surface is used to immobilize the aptamer and an electrochemical transducer is held for monitoring the aptamer-target interaction, by detecting current or potential changes that take place at the transducer/bioreceptor interface/surface (Hong et al., 2012; Monošík et al., 2012; Sadik et al., 2009; Thévenot et al., 2001).

Different voltammetry techniques can be used among voltammetric biosensors such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), square-wave voltammetry (SWV) and alternating current voltammetry (ACV). The application of a potential to a working electrode versus a reference electrode is the characterization of the voltammetric biosensors. The corresponding current is a result of electrolysis by means of an electrochemical reduction or oxidation at the working electrode. In the voltammetric biosensors, the potential is scanned over a set potential range and both the current and potential are recorded and measured. In case it's an amperometric or voltammetric biosensor, the recorded current response is proportional to the concentration of the target molecule in the sample (Grieshaber et al., 2008; Kumar et al., 2012; Liu., 2000; Rahman et al., 2008; Ronkainen et al., 2010; Viswanathan et al., 2008).

Among the voltammetric aptasensors, those based on CV, DPV and SWV are the most frequently used. However, to evaluate the electrode surface, namely its reproducibility, stability, purity and repeatability, the CV technique can be used. It may also be used for cleaning and for monitoring the immobilization of aptamers on the electrode surface since it allows a fast visualization of the redox behavior over a wide potential range (Ferreira et al., 2011). Added to that, CV technique can also be used to study the performance of an electrochemical aptasensor during the detection of several molecules, such as proteins (Cheng et al., 2007; Meirinho et al., 2015; Wang et al., 2009; Yuan et al., 2011).

An exceptional advantage of voltammetric biosensors is the low related noise observed, hence providing reliable and reproducible data for the quantification of a target molecule, which can endow the biosensor with higher specificity and sensitivity (Labib et al., 2014).

Areas, where electrochemical aptasensors can be applied, are very diversified such as health like in clinical diagnostic and for therapeutic purposes, food industry and environmental monitoring for the detection of protein disease biomarkers, pathogens and small molecules,

respectively (Hayat et al., 2014; Hong et al., 2012; Kim et al., 2014; Labib et al., 2014; Liu et al., 2012; Palchetti et al., 2012).

Aptasensors has also been used in recent researches to detect other cancer biomarkers such as the development of an electrochemical sensor using the 76-nucleotide DNA ERα- aptamer for precise, cost-effective and rapid, detection of estrogen receptor alpha (ERα) expression in human breast cancer patients (Ahirwar et al., 2018). Indeed, the analysis of estrogen receptor (ERα) expression in breast carcinomas plays an important role in the determination of the endocrine responsiveness of tumours for systemic adjuvant therapy. Also, electrochemical aptasensors have been used to early detect prostate-specific and free prostate-specific antigens released by cancer cells (Parra et al., 2018). It is a simple and non-invasive methodology to detect prostate cancer.

1.5.2. Aptamers as bioreceptors

Aptamers are oligonucleotide or peptide molecules that bind to a specific target molecule. (≈ 12-80 nucleotides long) they are formed by single-stranded DNA or RNA, they possess unique binding characteristics to their targets, like high sensitivity, high affinity, specificity and ability to fold into numerous tertiary conformations (e.g. hairpin, G-quartet, stem-bulge, pseudoknot, T-junction) (Cho et al., 2009; de-los-Santos-Álvarez et al., 2008; Famulok and Mayer, 2011; Lakhin et al., 2013; Meyer et al., 2011; Radom et al., 2013).

They are usually created by selecting them from a large random sequence pool. This selection methodology is termed SELEX (systematic evolution of ligands by exponential enrichment) (Cheng et al., 2009).

1.5.3. SELEX: Methodology to select aptamers

The SELEX methodology also referred to as *in vitro* selection or *in vitro* evolution, is a combinatorial chemistry technique in molecular biology for producing aptamers, it starts with a chemically synthesized random oligonucleotides library (up to 10^{15} different sequences). As presented in Figure 4, the selection process can be divided into 3 steps, namely binding, separation/partitioning and amplification, which are iteratively repeated to obtain nucleotides having improved binding ability towards the desired target. After a number of cycles (generally 5–15 cycles), the sequences obtained are cloned, sequenced and their binding affinity, secondary structure and Gibbs energy are evaluated in order to select the aptamers with high specificity and affinity to the target molecule (Darmostuk et al., 2015; Dua et al., 2011; Radom et al., 2013; Santosh et al., 2014; Song et al., 2012; Syed et al., 2010; Vikesland et al., 2010).

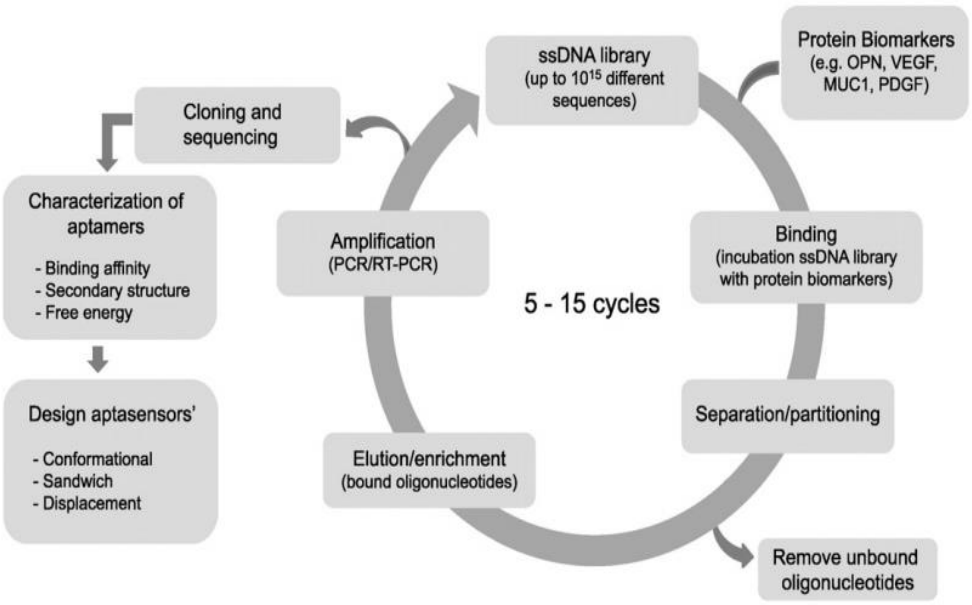

Figure 4. Systematic Evolution of Ligands by Exponential Enrichment (SELEX) methodology (Meirinho et al., 2016).

Up to now, a variety of aptamers exhibiting high selectivity and affinity towards relevant protein disease biomarkers have been reported, thus allowing the fabrication of new, simple and sensitive diagnostic methods to determine such proteins in standard solutions and in complex samples such as blood and serum. The use of electrochemical aptasensors is particularly interesting given their simplicity, sensitivity, specificity and suitability for the detection of low levels of protein disease biomarkers using several available techniques including CV, SWV and DPV.

In this work, we are going to use a DNA aptamer to detect osteopontin for the reason that DNA aptamers are suitable for designing reusable aptasensors while RNA aptamers allow single-shot measurements since they are more susceptible to nucleases attack (Sassolas et al., 2009; Strehlitz et al., 2008).

1.6. Objectives

The aim of this work is to assess the enhancement performance of a novel DNA aptasensor for osteopontin detection, based on a new DNA aptamer, which was previously obtained using SELEX methodology. For that, electrochemical tools were used, being aimed to characterize the performance of a new aptasensor using cyclic voltammetry. The objectives are to assess the electrochemical detection of human osteopontin using different screen-printed gold electrodes

and the selected aptamer to determine the detection and quantification limits. The evaluation of repeatability assays in order to calculate the coefficient of variation. The study of the specificity of the DNA aptasensor to other proteins using thrombin, lysozyme and bovine serum albumin which were evaluated by a previous research as possible interferents.

2. Material and methods

2.1. Reagents

Sodium chloride (NaCl), potassium chloride (KCl) and sodium hydrogen phosphate (Na2HPO4) were obtained from Panreac. Potassium hexacyanoferrate (III) [$K_3Fe(CN)_6$] and potassium hexacyanoferrate (II) [$K_4Fe(CN)_6$] were acquired from Acros Organics and potassium dihydrogen phosphate (KH2PO4) from Merck. 3,3-dithiodipropionic acid (DPA), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine (ETA), and streptavidin were purchased from Sigma-Aldrich. Recombinant human OPN (rhOPN), was obtained from R&D systems and manipulated according to the manufacturers' specifications.

2.2. Solutions

Stock solutions of 100 mM of NHS, 200 mM of EDC, additionally to the stock solution of 1 mg/ml streptavidin in PBS (pH 7.4) were prepared and stored at -20 ºC before use. Phosphate buffer saline (PBS) was prepared to contain 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, with adjusted pH 7.4. The redox probe was prepared in order to obtain a solution with a concentration of 5 mM $K_4Fe(CN)_6$: $K_3Fe(CN)_6$ (1:1) and with 10 mM of KCl in 100 mL of PBS, at pH 7.4. Stock solutions of 100 mM of ETA and 200 nM of DPA were prepared and stored at 4 ºC. Stock solutions of protein were prepared according to manufacturer specifications and stored at -20 ºC. The protein working solutions were prepared and diluted to a concentration of 200nM with PBS buffer and stored at 4 ºC before use.

2.3. Apparatus and software

The pH was measured using a pH meter (iHANNA instruments pH 211). A DropSens potentiostat (µStat 200, reference DRP – STAT200) was used to record CV signal profiles (Figure 5). The disposable screen-printed gold electrodes (SPGEs) chip (reference C223AT - high temperature curing ink, DropSens, S.L., Spain) used had a gold working electrode with a diameter of 1.6 mm, as well as a silver pseudo-reference electrode and a gold counter electrode (Figure 6). A DropSens connector (µStat cable connector, reference CAST, DropSens, S.L., Spain) was used as an interface between the SPGEs and the DropSens potentiostat (Figure 5). The Drop View 2.0 software was used to study the electrochemical processes taking place at the electrode's surfaces.

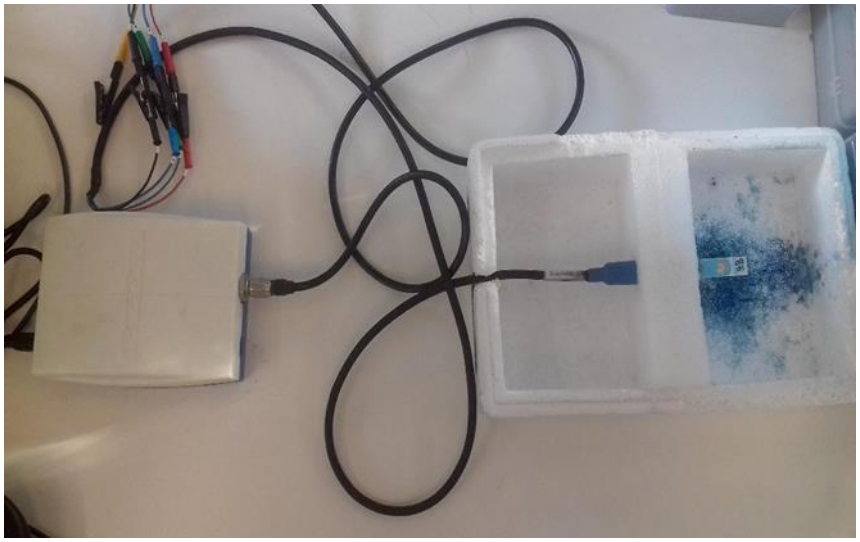

Figure 5. Potentiostat-Galvanostat device, cable connector and screen-printed gold electrode.

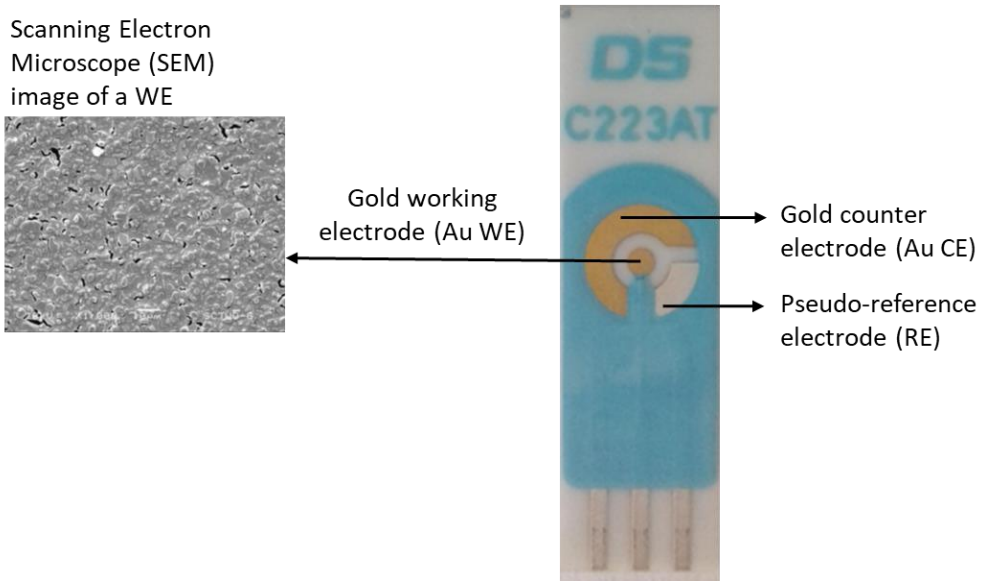

Figure 6. Screen-printed gold electrode (reference C223AT, DropSenses).

2.4. DNA aptamer

The DNA aptamer (C10K7) against human OPN has been isolated through the SELEX methodology as described before. The sequence of the biotinylated DNA aptamer that was synthesized by Integrated DNA Technologies (Belgium) is as follows: 5'- Biotin- CAT AAC-ACG GTA ATT GTG TGA ACC GCT GTG -3'. The stock solutions of the synthetic oligonucleotides were prepared with distilled water. The working DNA aptamer solution was prepared every day by dilution to the desired concentration (8 nM) using PBS.

2.5. Immobilization of the DNA aptamer on a gold surface

The use of an efficient immobilization method and a specific aptamer are required to construct an electrochemical-based aptamer biosensor with high affinity for rhOPN. The biotinylated DNA aptamer was immobilized onto the screen-printed gold electrode (SPGE) by a streptavidin-biotin interaction. To get to immobilize the aptamer the following steps were made: first of all, the electrode was electrochemically cleaned by successively cycling the electrode using three sulfuric acid solutions (0.5 M H_2SO_4, 0.01 M KCl/0.1 M H_2SO_4 and 0.05 M H_2SO_4) under electric potential in the range of -0.3 to 1.5 V, and with a scan rate of 100 mV/s. The cleaning procedure ended when a representative cyclic voltammogram of a clean gold electrode was obtained. In order to ensure the uniformity of the working electrode, $[Fe(CN)_6]^{-3/-4}$ solution was used to verify if the typical CV voltammogram of homogenized gold electrode was reached. After that, the self-assembled monolayer (SAM) was spontaneously formed through incubation of 200 mM of DPA for 30 min. After washing with deionized water, the working electrode was treated, during 1 hour, with the same volumes of 1 mM of NHS and 100 mM of EDC in order to activate the carboxyl groups, so that they can bind with the amino-terminal of streptavidin. After that, the working electrode was incubated overnight with streptavidin solution at 4 ºC. After washing with PBS 1×, the working electrode was then exposed to ETA (100 mM, pH 8.5) for 20 min in order to block any remaining activated -COOH groups. Finally, the DNA aptamer in PBS buffer (pH 7.4) was attached to the modified gold surface using the streptavidin-biotin interaction for 40 min. All reactions were carried out at room temperature.

2.6. Osteopontin incubation

Standard solutions of rhOPN in PBS buffer (200nM) were dropped on the working electrode and incubated for 1 hour. After washing the electrode with PBS 1×, 60µL of $[Fe(CN)_6]^{-3/-4}$ solution was dropped on the electrodes chip until all three electrodes were immersed. Then, cyclic voltammetry (CV) was performed and an electrochemical signal was generated due to the aptamer-target molecule interaction in the presence of $[Fe(CN)_6]^{-3/-4}$, under a potential range of -0.3 to 1.5 V, and with a scan rate of 50 mV/s.

2.7. Cyclic voltammetry analysis

After osteopontin incubation, the electrochemical analyses were done as those performed after aptamers immobilization and the obtained current peak was recorded. The electrochemical analysis was performed at room temperature. The current decrease was calculated through

relative current change, (ΔI %), the difference between the current values of the voltammogram oxidation peak recorded after analyzing the protein solution and that recorded after aptamer immobilization divided by this last one, using the equation:

$$\Delta I\ \% = (I_0 - I_1)/ I_0 \times 100$$

where, ΔI is the relative current change; I_0 and I_1 represent the current measured using the information regarding the oxidation peaks before and after the sample treatment, respectively.

The detection limit (LOD) and quantification limit (LOQ) were calculated based on the linear relationship obtained between different rhOPN concentrations and ΔI % values of the oxidation peak current. The detection limit is calculated by the equation LOD = 3.3(SD/b) and the quantification limit is calculated by the equation LOQ = 10(SD/b), where SD is the standard deviation of the intercept and b is the average slope of the regression line (Ermer and Miller, 2005). Additionally, repeatability assays were studied in order to evaluate the closeness of the agreement between the results of osteopontin immobilization made the same day with the same protein concentration but with different SPGEs. Finally, the specificity of the DNA aptasensor to other proteins was also evaluated by means of the electrochemical signal generated from the interaction between the DNA aptamer and a solution of each protein (THR, LYS and BSA; 200 nM), under similar experimental conditions.

3. Results and discussion

3.1. DNA aptamer selection

As mentioned, aptamers are short single-stranded oligonucleotides that are able to bind to specific target molecules with high affinity and specificity. Binding occurs due to their specific and complex three-dimensional shapes that are characterized by stems, loops, bulges, hairpins, pseudoknots, triplexes, or quadruplexes. The aptamer-target binding is the result of structure compatibility, stacking of aromatic rings, electrostatic and van der Waals interactions, hydrogen bonding, or a combination of these effects (de-los-Santos-Álvarez et al., 2008; Lönne et al., 2014; Stoltenburg et al., 2007; Strehlitz et al., 2012). In this work, a DNA aptamer (C10K7) previously isolated by the research team (Meirinho, 2016; Meirinho et al., 2017) through the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) methodology towards rhOPN was selected. Several sequences (aptamers) were isolated and their applicability was theoretically evaluated taking into account the sequence' Gibbs energy (ΔG values, being the lowest one the most desirable, since it would correspond to the highest stability), their homology and capability to form stable secondary structures considering the aptamer full-sequence (70 nucleotides, nt) or the random region (30 nt).

According to the previous research (Meirinho, 2016; Meirinho et al., 2017) 19 clones were isolated and sequenced. The sequences that exhibited the lowest ΔG values for the full sequence with conserved regions (70 nt) were C10K2, C10K7, C10K3, C10K5, C10K9, C10K15 and C10K18. Considering the random region (30 nt), the sequences with the lower ΔG values were C10K2, C10K7 and C10K15 and these were found to form a stable secondary structure (Meirinho, 2016; Meirinho et al., 2017). A former sequence (C10K2) was already studied showing a satisfactory performance regarding the detection and quantification of osteopontin (Meirinho, 2016; Meirinho et al., 2017). In this work, the DNA aptamer C10K7 (Figure 7) was selected because of its low ΔG value, homology and ability to form stable secondary structures considering the aptamer full-sequence (70 nucleotides, nt) or the random region (30 nt).

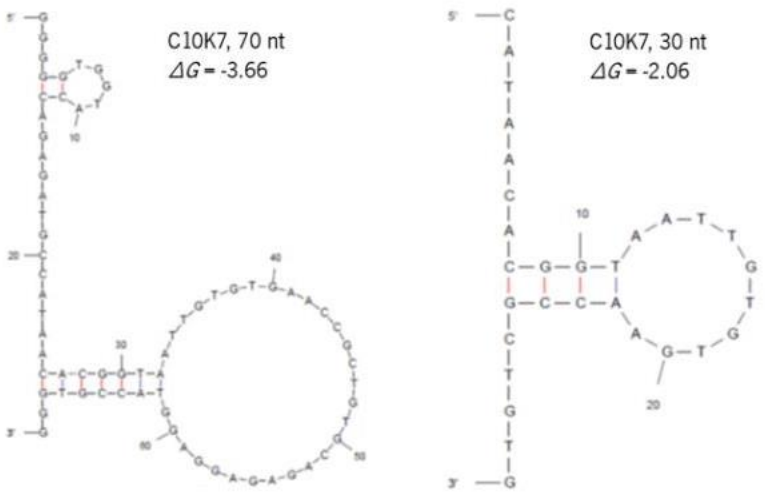

Figure 7. Secondary structure of the selected C10K7 DNA aptamer. The DNA aptamer sequence of C10K7 with 70 and 30 nucleotides were previously analyzed using the MFold software (Meirinho, 2016).

3.2. Electrochemical evaluation of the electrode surface

The voltammetry assays were conducted after each preparation step of the aptasensor in order to verify the electrochemical behavior of the electrode surface, being the first phases the cleaning steps followed by the immobilization steps. The CV is considered a convenient and valuable tool to monitor the modifications of the surface of the electrodes. It allows measuring the electron transfer between the solution species and the electrode surface that must occur by tunneling either through the barrier or through the defects in the barrier (Olowu et al., 2011).

Figure 8 shows the cyclic voltammograms recorded after each preparation step, using a 5 mM solution of $[Fe(CN)_6]^{3-/4-}$ in PBS solution as the electrochemical probe. As it can be observed from the cyclic voltammograms shown in Figure 8.A, a quasi-reversible voltammetric profile was recorded with the screen-printed gold electrodes (SPGEs: DS-C223AT) before any treatment (i.e., bare Au electrode full line in Figure 8.A), with potential variation between cathodic and anodic peaks of 100 mV (ΔEp) and with similar cathodic and anodic peaks current intensities (~25 μA). The current response decreased significantly after the cleaning steps (i.e., AFC dashed line in Figure 8.A), which included the use of different H_2SO_4 solutions. The observed decrease may be due to achieving a homogeneous electrode surface. After the formation of the DPA self-assembled monolayer on the electrode surface (i.e., DPA dot line in Figure 8.A), an expected decrease of the peaks' current intensities was observed, as well as an increase of ΔEp, which may be due to the electron transfer blocking. Afterward, the electrode

surface was activated with EDC-NHS that leads to the activation of the carboxyl groups and results into an increase of the peaks current intensities (Figure 8.B). The activated carboxylic groups on the electrode surface facilitate the binding of the streptavidin amine's groups. Comparing with the previous step, the streptavidin layer in the electrode surface increased the ΔEp value and decreased once more the current peaks' intensities. After exposing the working surface electrode to ETA, with the aim to block any remaining activated carboxyl groups, the cyclic voltammogram obtained showed a decrease in the ΔEp and of the current peaks' intensity as compared with the previous one, which may be due to some non-specific blocking of the remaining free carboxylic groups.

After the immobilization of the DNA aptamer on electrode surface (C10K7), a typical cyclic voltammogram (Figure 8.C) was recorded showing lower current peaks intensities when compared to the previous steps. This decrease can be due to the negative charges of the aptamer backbone phosphate group and $[Fe(CN)_6]^{3-/4-}$ redox probe, the electrostatic repulsive interaction is able of blocking the electron transfer (Bang et al., 2005). This result clearly demonstrates the effective immobilization of the aptamer onto the working electrode surface. After rhOPN incubation, the difference between the cyclic voltammograms obtained after the aptasensor preparation and after its incubation with a standard rhOPN solution (50.05 nM) is significant (Figure 8.C, dashed and dot lines, respectively). Actually, for the rhOPN analysis, the cyclic voltammogram showed the highest potential variation between peaks and the lowest current peaks when compared to all the previous steps. The decrease of peak current intensities demonstrates the aptamer-rhOPN complex formation, by means of the specific recognition increased hindered electron transfer. This "signal-off" sensing mechanism in the current, also called negative readout signal, after aptamer-target interaction may be attributed to a change of the aptamer conformation. The abovementioned voltammetric changes were similar to those previously described in the literature during the cleaning and immobilization steps of other SPGEs for the detection of osteopontin with RNA and DNA aptamers (Merinho et al., 2015, 2017; Meirinho, 2016).

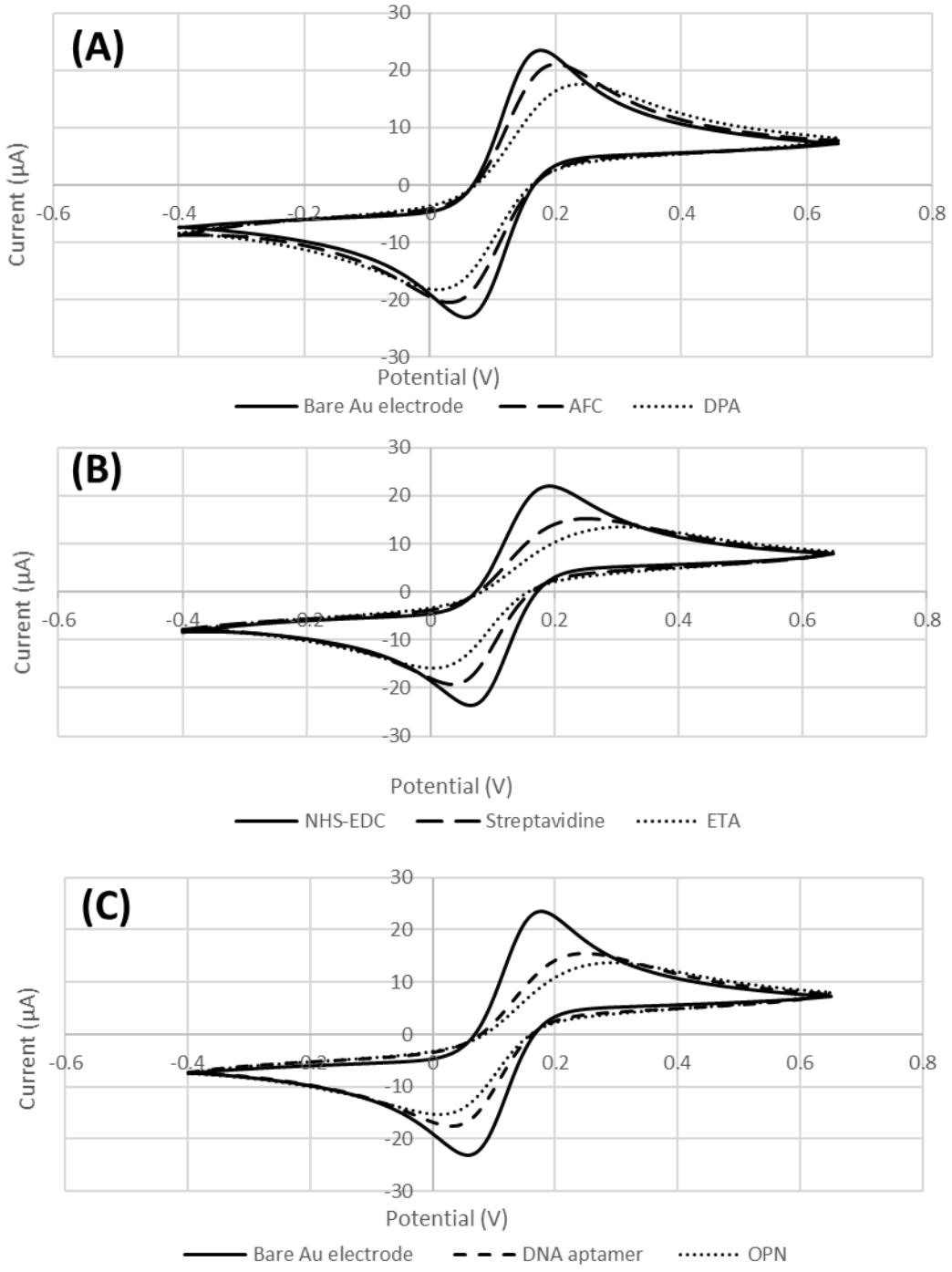

Figure 8. Cyclic voltammograms of 5 mM $[Fe(CN)_6]^{3-/4-}$ probe in PBS buffer solution of pH 7.4 at a scan rate of 100 mV/s for all aptasensor preparation steps: bare Au electrode, cleaning, DPA, EDC/NHS, streptavidin, ETA, DNA aptamer and DNA aptamer-rhOPN protein.

3.3. Repeatability

For each rhOPN concentration evaluated, two SPGEs (DS-C223AT) were used for immobilization and if the results obtained with the two chips were not similar a third SPGE was used to confirm the results. Figure 9 shows two voltammograms obtained after the incubation of a rhOPN solution with the concentration of 50.05 nM, with two different chips, being the assays carried out on the same day and under the same operating conditions. A coefficient of variation of 7% was calculated using the standard deviation and the mean ΔI (%) values showed the satisfactory repeatability of the method. Indeed, Figure 9 shows an almost perfect superposition of the two voltammograms, which have similar cathodic and anodic peaks current intensities and similar relative current change (11.7% and 12.9%).

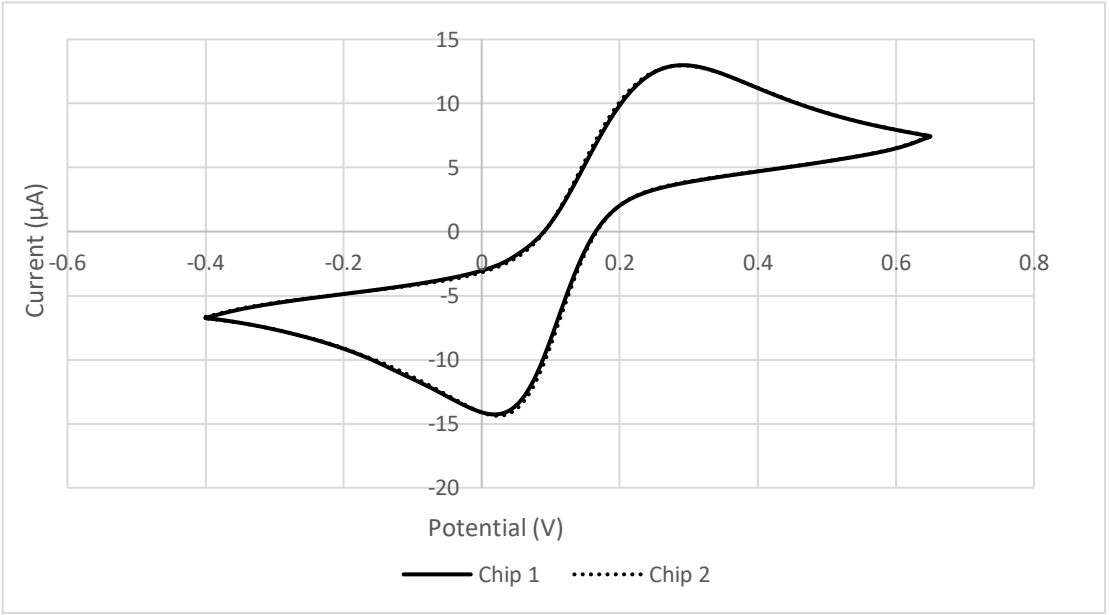

Figure 9. Intra-day repeatability assays: cyclic voltammograms after rhOPN immobilization (50.05nM) for two different SPGEs.

3.4. Electrochemical detection of human OPN

The performance of the aptasensor was evaluated under specific experimental conditions, taking into account its response (ΔI% values) against standard solutions that contain different concentrations of rhOPN. Each rhOPN determination was carried out using different SPGEs. The cyclic voltammograms of the redox probe $[Fe(CN)_6]^{3-/4-}$ solution showed an electrochemical response that is related to the modification of the aptamer surface and, in this case, to the level of aptamer–rhOPN interactions, which depend on the rhOPN concentrations.

The CV assays showed a decrease in the anodic and cathodic current responses (Ipa and Ipc) as a consequence of the increase of the rhOPN concentration. In Figure 10 it is shown the response obtained (ΔI% values) as a function of the rhOPN concentration for a dynamic concentration range of 1.5 to 201 nM. The results showed an increase of the ΔI% values with the increase of the rhOPN concentration, being two distinct linear zones identified, one for rhOPN concentration up to 12.5 nM and another for concentrations ranging from 12.5 and 201 nM. Thus, two calibration curves were established by linear regression (Figures 11 and 12):

$\Delta I\ (\%) = 4.8(\pm 0.1) + 0.28(\pm 0.02) \times [\text{OPN, nM}])$ for $1.5\ \text{nM} \leq [\text{OPN}] \leq 12.5\ \text{nM}$

$\Delta I\ (\%) = 6.8(\pm 0.2) + 0.107(\pm 0.002) \times [\text{OPN, nM}])$ for $12.5\ \text{nM} \leq [\text{OPN}] \leq 201\ \text{nM}$

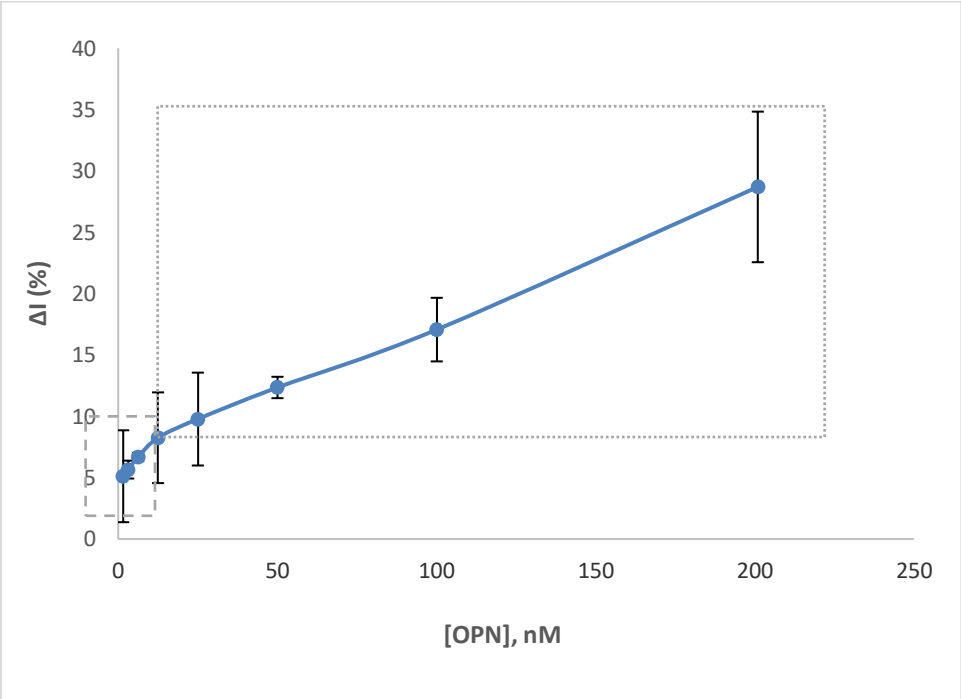

Figure 10. Electrochemical aptasensor sensitivity analysis of rhOPN using an aptamer-immobilized gold working electrode.

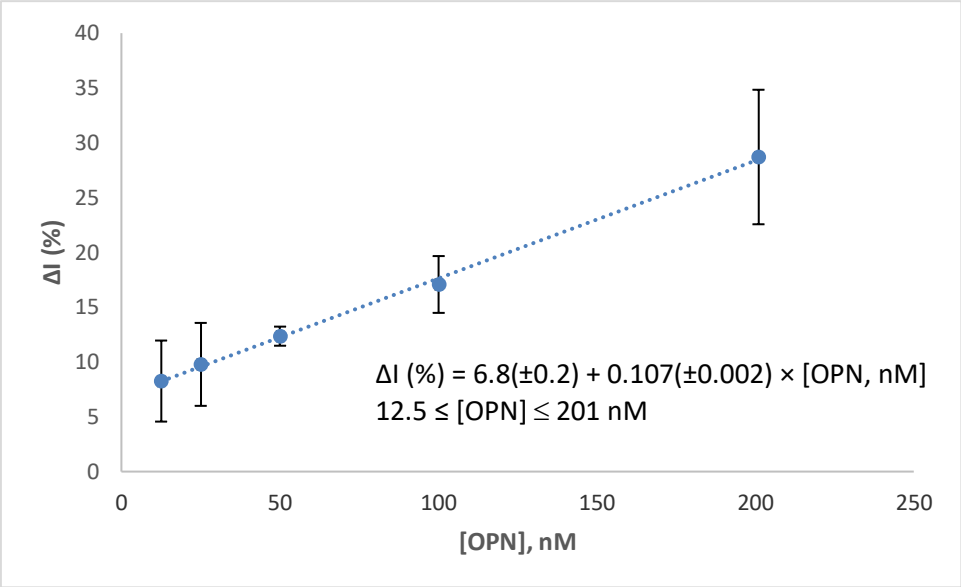

Figure 11. The linear relationship between the ΔI (%) and the rhOPN, in the range of 12.5 to 201 nM.

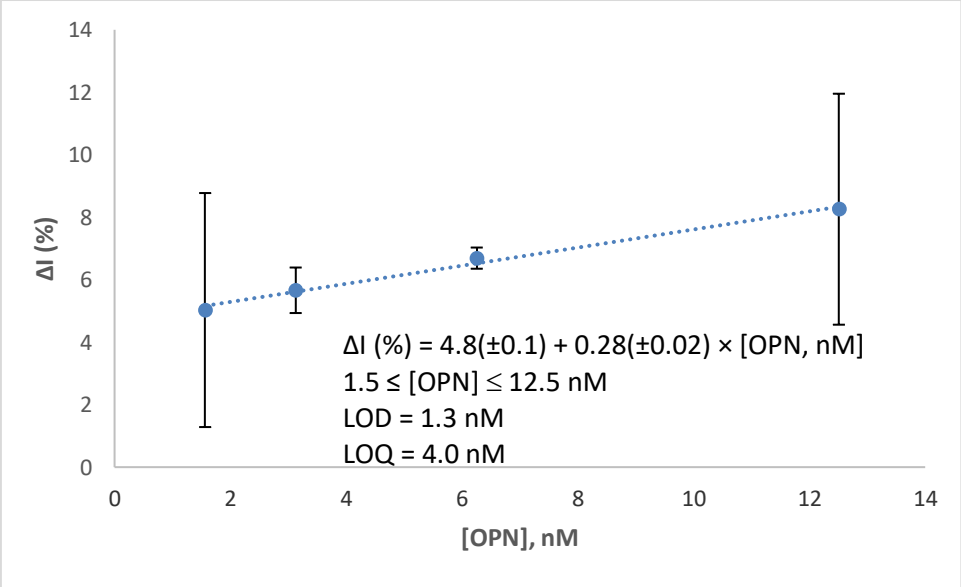

Figure 12. The linear relationship between the ΔI (%) and the rhOPN concentrations, in the range of 1.5 to 12.5 nM.

The linear determination coefficients (R^2) were of 0.9940 and 0.9984 for the lowest and highest concentration ranges, being calculated the LOD and LOQ values based on the regression line coefficients of the calibration curve shown in Figure 12. The calculated LOD and LOQ values were equal to 1.3 nM and 3.95 nM (i.e., 84.5 ng/mL and 256.75 ng/mL, assuming a molecular weight of 65 kDa), respectively. These LOD and LOQ values were improved compared to all the previous work using CV and equal to the result previously found with SWV (Table 5), which is a more sensitive voltammetric technique. This finding suggests that a future analysis

with SWV and the DNA C10K7 aptamer may allow a diminishing of the reported LOD and LOQ values and so foresee a possible practical application of this electrochemical tool.

Table 5. Comparison of the analytical characteristics of the actual proposed DNA aptasensor with the previously reported methods for rhOPN detection.

Detection method	Characteristics of the method	Limit of detection (nM)	Limit of quantification (nM)	References
Electrochemical aptasensor SWV	RNA aptamer immobilization	10.7 ng/mL	Not mentioned	(Tuck et al., 2007)
Electrochemical aptasensor CV	RNA aptamer immobilization	3.7 ± 0.6	11 ± 2	(Scatena et al., 2007)
Electrochemical aptasensor SWV	DNA aptamer immobilization	1.4 ± 0.4	4.2 ± 1.1	(Meirinho, 2016)
Electrochemical aptasensor CV	DNA aptamer immobilization	2.6 ± 0.3	7.9 ± 1	(Meirinho, 2016)
Electrochemical aptasensor CV	RNA aptamer immobilization	3.7 ± 0.6	11 ± 2	(Meirinho et al., 2015)
Electrochemical immunosensor EIS	Antibody immobilization	0.17	Not mentioned	(Wai and Kuo., 2007)
Electrochemical aptasensor CV	DNA aptamer immobilization	1.3	4.0	This Work

Taking into consideration the reported range of OPN concentrations found in plasma samples from patients with breast cancer (up to 290 ng/mL) with either metastatic or recurrent breast cancer (Bramwell et al., 2014) the proposed aptasensor could be applied in both OPN detection and quantification. Also, as mentioned previously, the future application of other voltammetry techniques, such as SWV and DPV, could increase the sensitivity of the proposed analysis. Actually, the SWV compared to CV is a technique that presents a broader dynamic range and lower limit of detection. Also, SWV enables a faster analysis added to less consumption of electroactive compounds, as a consequence it reduces the blocking problems of the electrode surface (Dogan-Topal et al., 2010). Eventually, the use of aptamers as bioreceptors instead of

antigen or antibody (immunological detection) makes this method less expensive and an interesting commercial alternative. Added to that, the use of aptamers presents some advantages like ease and a reduced cost of production.

3.5 Specificity of the DNA aptasensor to other proteins

The specificity of an aptamer towards its target molecule has a huge importance in the evaluation of the aptasensor performance and it can be evaluated by comparing the DNA aptamer binding capability with the specific protein (rhOPN in this case) and with non-specific proteins. Non-specific binding to the aptasensor cause high background signals which leads to the decrease of its performance. Three proteins (THR, BSA and LYS) were evaluated as possible interferents similar to previous studies (Meirinho et al., 2015, 2017; Meirinho, 2016). These proteins were chosen to take into account their isoelectric points and molecular weights, as well as their distinctive features that may affect the aptasensor performance (Meirinho, 2016). Thrombin (THR) was used because the OPN has a conserved thrombin cleavage domain (RSK (arginine168-lysine170) adjacent to the RGD domain). It is also a secreted serine protease found in the human blood (Beausoleil et al., 2011; Gursoy et al., 2010). The bovine serum albumin (BSA) is inert and similar to the human serum albumin (HSA), which also is present in high concentrations in human serum samples (Gokulrangan et al., 2005). Finally, lysozyme (LYS) was used as a control protein since it possesses the lowest molecular weight and highest peak current intensity compared with all the other proteins studied, it is also known to non-specifically bind to nucleic acids (Gokulrangan et al., 2005). The relative current response ($\Delta I\%$) for the interferent proteins tested was found to be negligible as compared to the specific binding response obtained with rhOPN as shown in Figure 13. The mean relative current intensity response for THR, BSA, LYS and OPN was 7.16%, 16.65%, 16.21% and 28.7% respectively. The DNA C10K7 aptasensor showed a better selectivity for THR but a lower selectivity to BSA and LYS when compared to the results obtained with another DNA aptasensor previously reported (Meirinho et al., 2015). The results also showed that the DNA aptamer had a good sensitivity to rhOPN when compared with the proteins BSA, LYS and THR. However, their ΔI % values cannot be neglected especially for LYS and BSA. Finally, these results suggest that this aptasensor still could be used to detect rhOPN in biological samples such as plasma, blood or serum.

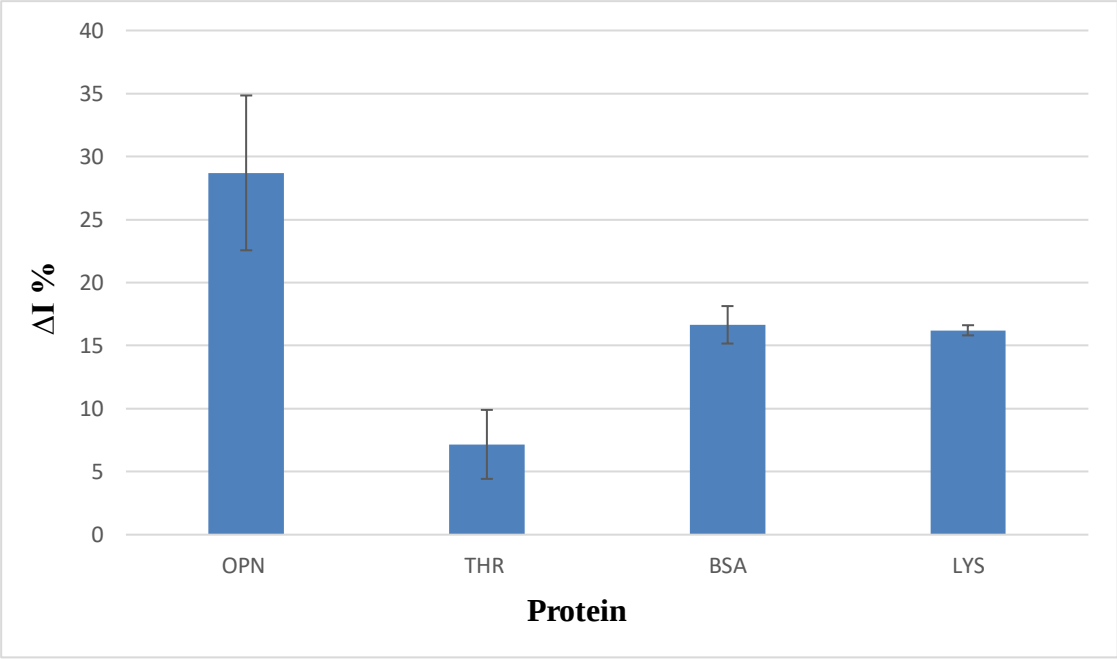

Figure 13. The relative current response (ΔI %) to non-specific proteins (200 nM): THR-thrombin, BSA – bovine serum albumin and LYS – lysozyme and for specific-protein rhOPN – human osteopontin. Error bars correspond to the relative standard deviation.

4. Conclusion and perspectives

The aim of this work was to assess the enhancement performance of a novel aptasensor for the detection and quantification of human OPN, in order to conceive the development of new strategies for the early detection and diagnosis of different types of cancer.

Electrochemical aptasensor devices have been widely explored for the detection of various proteins disease biomarkers, mostly due to their interesting features including high sensitivity, low-cost and simple preparation, reusability and rapid response. In this thesis, a screen-printed gold electrode (SPGE) with a gold working electrode (1.6 mm of diameter), was used as it allows reducing the amount of reagents and solutions required and miniaturizing the biosensor device. Additionally, aptamers have been reported as suitable bioreceptors due to their easy chemical production, high thermal stability and possibility to be modified with chemical groups that are useful for the functionalization of the electrode surfaces.

In this work, a new DNA aptamer (C10K7), previously isolated through SELEX methodology by the research team, was selected and used. The results obtained are satisfying as the aptasensor showed high sensitivity, specificity and repeatability towards the detection of rhOPN. The detection and quantification limits are lower than those reported in previous works using CV and equal to those reported using SWV as it is considered to be a more sensitive tool compared to CV. In conclusion, the development of electrochemical aptasensor using this aptamer can be a simple and efficient strategy for the detection of relevant biomarkers, for the diagnosis and therapy monitoring, as well as a promising alternative to directly analyze complex samples such as biological fluids like blood or plasma.

Despite the interesting and relevant results herein obtained, additional work is still needed to improve this study, like the use of SWV, a more sensitive tool, instead of CV as it may lead to a better detection and quantification limit and the evaluation of the human OPN detection by this DNA aptasensor in biological fluids such as blood and plasma.

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