INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO
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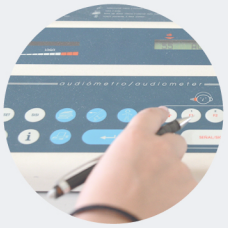


DETECÇÃO DE POLIMORFISMOS ASSOCIADOS À FARMACOGENÉTICA CARDIOVASCULAR através de GENOSSENSORES ELETROQUÍMICOS

AYA MARSHAHA
Novembro de 2021

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Engenheiro(a) Biomédico(a) pelo Instituto Superior de Engenharia do Porto

Dissertation presented at the Superior Institute of Engineering of Porto to obtain a Master's degree in Biomedical Engineering

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Institutional support

This work received financial support from project MultiSNPsensor aplicado à farmacogenética cardiovascular, supported by national funds from Fundação para a Ciência e a Tecnologia (FCT) e a Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasil) and UIDB/50006/2020 with funding from FCT/MCTES through national funds.

Acknowledgement:

I would first like to thank my supervisors Dr. Fátima Barroso, Dr. Júlia Magalhães, and Prof. Valentina Domingues. For their relentless desire to assist me in bringing this study to a successful conclusion. I thank them deeply for all the guidance and unconditional support they have given me throughout this project. They were ready at any time to help me or answer any questions I had. Without them I could not, under any circumstances, defend my master's thesis.

I would also like to thank Stephanie Morais and Carla Rodrigues for my valuable assistance with laboratory procedures and for discussing any aspect of this study with me.

I would also like to thank the Analytics and Chemical Reactions group team | The Network of Chemistry and Technology (GRAQ | REQUIMTE) of the Higher Institute of Engineering in Porto to welcome me with open arms and for all the support and complicity offered.

I would also like to thank Dr. Helena Barocco in charge of the scholarship for giving me this great opportunity and without her support I would never be here

I would also like to leave a word of thanks to my family and husband for their support and patience in times of great frustration and grief. Listen to me and give me the strength to move forward.

Finally, I want to thank my friends who have made this walk with me during these two years. I refer to Nair Araujo, Daniela Oliveira, Tomás Sousa and Teresa Ferraz. they helped me a lot, answered most of my questions during the two years thank you so much for everything.

Abstract

Cardiovascular diseases (CVD) are one of the leading causes of death worldwide. To prevent CVD events and further loss of life anticoagulants, like warfarin, are prescribed and administered to patients. Nevertheless, warfarin presents a strict therapeutic window which can lead to other CVD complications or treatment prevention.

Studies have shown that genetic determinants, such as single-nucleotide polymorphisms (SNP), can alter the function of enzymes involved in vitamin K's and warfarin's metabolism and action, causing interindividual differences in the drug response.

In this thesis, electrochemical genosensors for the detection of the vitamin K epoxide reductase complex (VKORC1) gene polymorphisms was developed and optimized. This device detects the electrochemical signal of the hybridization reaction between two complementary DNA sequences.

Analyzing public databases, two DNA target probes (52 bp) with the A and G nucleotide variants were selected and designed.

The construction of the genosensor was obtained in various steps (i) Sensorial phase: creation of the thiolated DNA and mercapto-hexanol mixed self-assembled monolayer (SAM) on the screen-printed gold electrode (SPGE) surface; (ii) Promotion of the DNA hybridization reaction in a sandwich format (to increase the selectivity) and (iii) Electrochemical detection of the hybridization reaction by evaluating the reduction reaction of tetramethylbenzidine/peroxide (TMB/H_2O_2) substrate.

After optimizing all of the analytical parameters, the calibration curves for both sequences were determined. A linear correlation between the analytical signal (electrochemical current) and the corresponding DNA target concentration were obtained in the 0.50 and 1.00 nM range.

Therefore, the developed electrochemical genosensor is a promising and low-cost analytical tool to determine and discriminate an individual's genotype and predict the adequate warfarin dose.

Keywords: Cardiovascular diseases, Electrochemical genosensors, Polymorphisms, Sandwich format hybridization, VKORC1, Warfarin.

Resumo

As doenças cardiovasculares (CVD) são uma das principais causas de morte. Para prevenir mais episódios cardiovasculares e vidas humanas anticoagulantes, como a varfarina, são prescritos e administrados aos pacientes. No entanto, a varfarina apresenta uma estrita janela terapêutica que pode levar a outras complicações cardiovasculares ou à prevenção do tratamento.

Estudos realizados demonstraram que variantes genéticas, tal como os polimorfismos num único nucleotídeo (SNP), podem alterar a função das enzimas envolvidas no metabolismo e na ação da vitamina K e da varfarina, provocando diferenças interindividuais na resposta ao fármaco.

Nesta dissertação foi desenvolvido e otimizado um genossensor eletroquímico para a deteção do complexo de epóxido de vitamina K (VKORC1). Este dispositivo deteta o sinal eletroquímico da reação hibridização entre duas sequências de ADN complementares.

Analisando bases de dados públicas, selecionou-se e desenhou-se duas sondas de ADN alvo (52 bp) com as variantes polimórficas A e G.

A construção do genossensor foi efetuada em várias etapas (i) Fase sensorial: criação de uma monocamada auto-organizada (SAM) mista constituída pelo ADN tiolado e o mercapto-hexanol (MCH) na superfície do elétrodo descartável de ouro (SPGE); (ii) Promoção da reação de hibridação do ADN em formato "sandwich" (para aumentar a seletividade) e; (iii) Deteção eletroquímica da reação de hibridação através da avaliação da reação de redução do substrato tetrametilbenzidina/peróxido (TMB/H_2O_2).

Após a otimização de todos os parâmetros analíticos, determinou-se as curvas de calibração para ambas as sequências. Obteve-se uma correlação linear entre a corrente eletroquímica e a concentração de ADN alvo no intervalo de 0,50 e 1,00 nM.

Portanto, o genossensor eletroquímico desenvolvido é uma ferramenta analítica promissora e de baixo custo para determinar e discriminar o genótipo de um indivíduo e prever a dose de varfarina adequada.

Palavras-chave: Doenças cardiovasculares, Genossensores eletroquímicos, Hibridação em formato em sandwich, Polimorfismos, VKORC1, Varfarina.

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ABBREVIATIONS

3D – three-dimensional

ADR – adverse drug reaction

ANTI-FITC-POD –anti fluorescein peroxidase

AU – gold

AU-S – gold-sulfur

AuSPGE – gold screen-printed gold electrodes

CVD – cardiovascular diseases

CYP1A1 –cytochrome P450 1A1

CYP1A2 – cytochrome P450 1A2

CYP2C19 – cytochrome P450 2C19

CYP3A4 – cytochrome P450 3A4

DNA– deoxynucleic acid

DNA-AuSPEs– homogenous hybridization onto the modified electrode

ELISA – enzyme-linked immunosorbent assay

GGCX –gamma-glutamyl carboxylase

LOD– limit of detection

LOQ– limit of quantification

MCH – 6-mercapto-1-1-hexanol

MENADIONE – methylated naphthoquinone

NP – nanoparticles

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

POD – horseradish peroxidase

S/B – signal / blank

SAM –self-assembled monolayer

SNPs–single-nucleotide polymorphisms

SPGE –screen-printed gold electrode

SSPE – saline sodium phosphate

TA – adenine

TG – guanine

TMP – 3,3 and 5,5 tetramethylbenzidine

VK – Vitamin K

VK1– phylloquinone

VK2 – menaquinones

VKORC1 – vitamin K epoxide reductase complex

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1. Introduction

Cardiovascular disease (CVD) is the major reason for death worldwide[1]. Molecular genetics and pharmacogenetic play an increasingly important role in the correct clinical management of patients [1]. For example, genetic testing can identify DNA variants that affect how patients metabolize drugs, making it possible to prescribe customized, safer, and more effective treatments, reducing medical costs and improving clinical outcomes [1].

Warfarin is an anticoagulant generally used to prevent blood clots from forming or increasing in size in the blood and blood vessels. It is usually prescribed to individual´s with certain types of arrhythmias, and people who have artificial heart valves. Since of the low therapeutic index of warfarin and frequent complications of prevention or treatment, significant differences in individual doses of warfarin are needed to achieve prophylactic and therapeutic ranges [2]. In fact, excessive anticoagulation can lead to significant local or systemic bleeding [3], which can be fatal [2][4]. On the other hand, there is a greater potential for thrombosis or embolism if patients do not receive optimal anticoagulants [5], [6]. The response to warfarin is influenced by patient-specific factors such as advanced age, lower body mass index (BMI), diet, and certain comorbidities [7],[4],[7]. Recent studies have focused on genetic determinants that alter the function of enzymes involved in vitamin K and warfarin metabolism and action [8], [9]. These mutations or polymorphisms also explain interindividual differences in the response to warfarin.

Recent studies have been reporting that genetic variants of vitamin K epoxide reductase complex (VKORC1) influence the response to warfarin and doses [9][12][13][14]. So, the genetic and pharmacogenetic information of the major cardiovascular diseases plays an important role in the identification of the cardiovascular risk factors and in the diagnosis and treatment of these conditions.

The DNA polymorphism is, by definition, a variation in the genome among the population with a minor allele frequency higher than 1% in the whole population. Polymorphisms are responsible for a variation in the human genome of around 1%, being the remaining percentage identical in every human being. These variations occur in all traits, at any segment of coding or non-coding DNA, attributing to all human beings their different characteristics [8].

The single-nucleotide polymorphisms (SNPs) are the most common polymorphisms in the human genome, having the frequency of one in 1000 base pairs, accounting for 10 million SNPs identified. As the name implies, SNPs are polymorphisms whose alteration is the substitution of one single nucleotide (adenine, guanine, cytosine, or thymine) in the DNA structure for another [9, 15, 16, 17].

1.1. Pharmacological Effect On Cardiovascular Health

Cardiovascular risk factors are highly prevalent and remain undiagnosed and inadequately treated [10]. For example, hypertension, a major risk factor for CVD, is a common disorder affecting approximately 950 million adults worldwide [15]. Most drugs are approved and developed based on their performance in large populations, and although they are guided by evidence from well-controlled clinical trials, they are less informative when treating individual patients. As a result, there is a growing need for methods to identify people who are most likely to benefit from pharmacological interventions and those who have the lowest risk of improving side effects when subjected to cardiovascular drugs.

1.1.1. Warfarin

Warfarin (Figure 1) is the most prescribed oral anticoagulant for the treatment and prevention of thrombotic diseases, including myocardial infarction, stroke, venous thrombosis, heart valve replacement and atrial fibrillation [16]. However, due to its narrow therapeutic index, it is one of the most common reasons for emergency room visits for adverse drug reactions [17]. In August 2007, the FDA considered that the accumulation of pharmacogenomic information was sufficient to justify a modification in warfarin labelling to highlight the potential relevance of genetic information to prescription evaluation [18].

The two main enzymes involved in the pharmacogenetics of warfarin are CYP2C9 (the CYP2C9) gene that is involved in metabolic clearance (pharmacokinetic relationship), and the C1 subunit of vitamin K epoxide reductase complex 2,3 (VKORC1) that recycles reduced vitamin K, which is essential. The post-translational carboxylation of coagulation factors is dependent on vitamin K II (prothrombin), VII, IX and X (pharmacodynamic relationship) [19]. Knowledge of the pharmacokinetics of warfarin (Figure 2) is useful in understanding the initial response to treatment as warfarin can be detected approximately 1 hour in plasma after oral administration, and peak concentrations appear within two to eight hours [20]. Warfarin is a racemic mixture (a substance containing an equal amount of left and right homologs of a chiral molecule) of -S and -R stereoisomers that will bind 99% to albumin. S-warfarin is converted by cytochrome P450 2C9 (CYP2C9) to inactive metabolites while R-warfarin is converted by Cytochrome P450 1A1 (CYP1A1), cytochrome P450 1A2 (CYP1A2), cytochrome P450 2C19 (CYP2C19), and Cytochrome P450 3A4 (CYP3A4). These enantiomers will perform as VK epoxidase reductase complicated inhibitor's, being the S-warfarin 3 to 5-fold more able than the R-warfarin enantiomer [21][22][23][24].

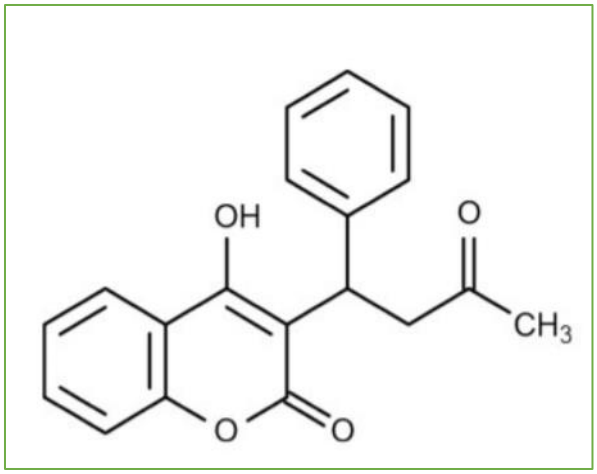

Figure 1 - Chemical structure for warfarin

The purpose of anticoagulant therapy is to give the lowest probable dose of the drug to prevent a clot from forming or expanding. Warfarin acts as an antagonist of Vitamin K, which is an essential component of the formation of coagulation factors II, VII, IX and X, as well as anticoagulant proteins C, S and Z. Anticoagulation occurs due to inactivation of the carboxylation of some glutamic acid residues by Vitamin K antagonists. This carboxylation process requires a reduced form of Vitamin K to allow the production of clotting factors and anticoagulant proteins. So, in the presence of Vitamin K antagonists, the rate of production of clotting factors and proteins will decrease by 30% to 50%, which allows anticoagulation [21][22].

The drug is metabolized in the liver and kidneys, with the production of inactive metabolites excreted in the urine and feces [20]. The half-life of racemic warfarin ranges from 20 to 60 hours, with a mean plasma half-life of about 40 hours, and the duration of effect from two to five days [20]. Thus, the maximum dose effect occurs up to 48 hours after administration, and the effect persists over the following five days. Due to genetic, clinical, physical, SES, and environmental factors, there is a known but not fully understood variable response among individuals to the administration of warfarin. The variance in patients' dose of warfarin can be significant from 0.6 mg/day to 15.5 mg/day. Individuals receiving warfarin treatment 45% to 63% of the time are usually within the therapeutic range (the higher the drug concentration the higher the potential for adverse effects, the lower the drug concentration is ineffective). However, the proportion of patients who show a positive response to warfarin in the treatment of CVD is significantly high, ranging from 50% to 75%. On the other hand, the proportion of patients who experienced adverse reactions is 15% [21][22][25][26].

Vitamin K is a fat-soluble vitamin with a methylated naphthoquinone (menadione) core and an aliphatic side chain at position 3, which can take two natural forms, Phylloquinone (VK1) and Menaquinones (VK2). Where Vitamin K in its miniature form plays an irreplaceable role in blood clotting because in the Vitamin K cycle clotting factors II, VII, IX and X are produced which are used in the coagulation cascade [27].

Initially the Vitamin K cycle (Figure 2) reduces the dietary form of Vitamin K. This reduction occurs through the action of quinone reductase which will in turn catalyze the donation of two electrons by NADPH, giving rise to Vitamin K hydroquinone. Then, Vitamin K hydroquinone will be the cofactor along with carbon dioxide and oxygen for the carboxyl step by giving its electrons to gamma-glutamyl carboxylase (GGCX). Which will allow GGCX electron acceptance to convert clotting factors and anticoagulation proteins into their functional forms. This occurs due to the addition of a carboxyl group to the end of the glutamic acid residues [22][28][29][30].

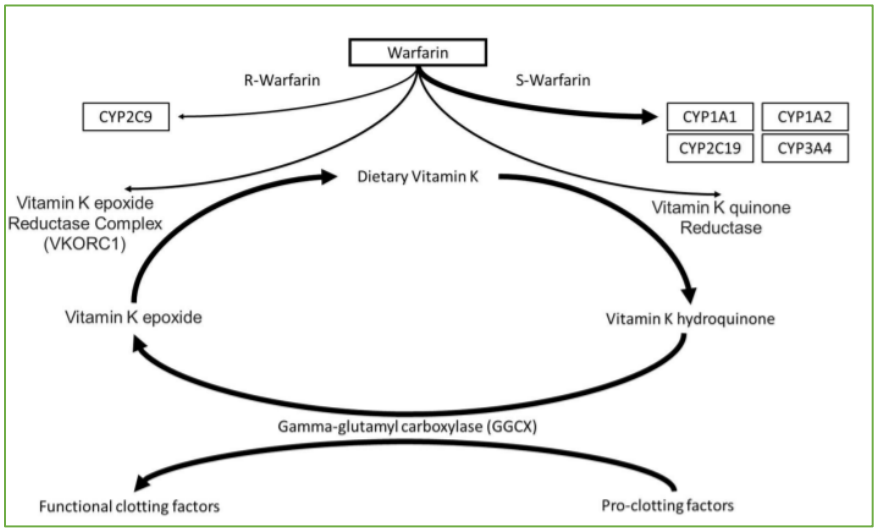

Figure 2 - Vitamin K cycle and respective warfarin effect on this process. Adapted from [32], [27].

1.1.2. Warfarin Pharmacokinetic

The CYP2C9 variants, Arg144Cys (CYP2C9*2) and Ile359Leu (CYP2C9*3) have been extensively studied due to their influence on *in vitro* and *in vivo* metabolic activities and allelic frequencies in different ethnic groups [31]. A large genome-wide association study [32] in over 1000 subjects showed that a polymorphism occurring in the CYP2C9 gene had a significant impact on warfarin therapy with a strong association between some of the polymorphisms and anticoagulant properties or bleeding complications. It has been shown that patients with CYP2C9*2 and CYP2C9*3 allele variants require lower doses of

warfarin to achieve an adequate anticoagulant status with minimal bleeding risk [33]. However, SNPs in CYP2C9 are only responsible for 6-18% of the total variance in the final warfarin dose) [16].

1.1.3. Warfarin Pharmacodynamic

It has been shown that patients with the VKORC1 A/A haplotype require lower doses of warfarin to achieve an adequate state of anticoagulation with minimal danger of blood loss [33]. However, VKORC1 haplotypes independently influence up to 30% of the total variance in final warfarin dose requirements. Subsequent studies demonstrated population differences in haplotype frequencies, suggesting that VKORC1 variants contribute to ethnic differences in warfarin doses [34]. In 2009, the International Consortium of Warfarin Genetics published a report demonstrating the usefulness and success of using a genotype-based approach to predict warfarin doses. It has been suggested that the pharmacogenetic approach was better at predicting the required warfarin dose than either the clinical algorithm alone or the fixed-dose approach [35]. However, genetics-based dosing cannot eliminate problems associated with clinical management such as fragmented care and socioeconomic factors, which impede the effective, frequent, and effective international normalized ratio measurement [36].

1.1.4. Conventional Methods For SNP Determination

The early diagnosis any disease-related risk factor, namely SNP, reduces the possibility, severity and any possible complications derived from a disease, playing an important role in the treatment outcome [37]. Thus, by identifying and comparing the variations in a population's DNA sequence, heritable genes relevant to specific phenotypic traits can help reduce or prevent diseases. Then again, advanced screening technologies are not yet commercially available for population genotyping. Ideally, a genotyping assay should be rapid, simple, robust, automated, highly accurate, sensible and cost-effective [38].

With the progress in genotyping technologies, many important drug-gene associations are being revealed. As the clinical value of predictive pharmacogenetic testing increases so does the search for individualized genotyping tool [39]. For this purpose, several conventional DNA genotyping techniques were developed (e.g. DNA sequencing, PCR-based methods, microarrays, mass spectrometry, flow cytometry). Among them, the polymerase chain reaction (PCR) and sequencing techniques have stood out [40]. Nevertheless, no single method is perfectly suited for every application [41].

Although, as we previously mentioned, statistical data reveals expenses and deaths arising from adverse drug reactions (ADR) that could be avoided. The success of personalized medicine depends on accurate diagnostic tests that identify patients who may benefit from targeted therapies.

Although the protein- and DNA-based assays of enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) are conventional and typically used as reference methodologies, they are not without their own drawbacks (i.e., laborious, expensive, and time consuming). Therefore, efforts have focused on developing low-cost methods that generate simple and fast measurements and easy to interpret data. Bioanalytical methods that have the potential for automation and microfabrication and are based on simple and portable detection systems (i.e., biosensors) represent a valuable tool for the detection and quantification of SNPs.

1.2. (Bio)sensors

A chemical sensor consists of a device that converts chemical information, from the concentration of a specific sample component to the analysis of the total composition and converts it into an analytically useful signal. Chemical sensors typically have two basic components connected in series: the chemical molecular recognition system (the acceptor) and the physical and chemical transducer. Biosensors are chemical devices in which the detection system uses a biochemical mechanism [42]. A biometric recognition system translates information from the biochemical field, usually the concentration of the analyte, into a chemical or physical output signal. The fundamental principle of a biosensor is based on the conversion of an analytical response, obtained through the biological actions of various substances, into a quantifiable signal. For this purpose, the analyte binds to the bioreceptor immobilized on the electrode by conventional methods (covalent, non-covalent or physical adsorption), which produces an electronic response that is converted into an electrical signal, amplified and then quantified. The signals obtained are recorded and transferred to a data storage device and later processed [43]. The performance of the biosensor depends mainly on its specificity and sensitivity to the biological reaction, in addition to the stability of the biomolecule. The advantage of these devices, compared to all other detection techniques, is that they can be miniaturized, allowing real-time and in situ analysis, in addition to being more sensitive, selective, easy to handle and without the need for specialized personnel [44].

1.2.1. Constituents of a Biosensor

Biosensors mainly consist of a biological component - the molecular recognition system - the transducer and the signal processing unit, as shown in the schematic diagram in (Figure 3) [45]. The purpose of these sensors is to identify/measure an analyte with minimal human interference. The biological component corresponds to the analyte - the substance to be detected (e.g., glucose, blood, urine, etc...) - and the bioreceptor - the molecule that specifically recognizes the analyte (e.g., enzymes, cells, aptamers, DNA, antibodies). The process of acquiring the signal (in the form of light, heat, a difference in pH, charge, mass, etc.) is called biological recognition and results from the interaction of bioreceptors with analytes, as these reagents are immobilized in the system [46]. The transducer serves as an interface, as it is the part of a biosensor that converts (the measurement of physical or chemical changes that occur during the interaction between an analyte and a bioreceptor) a biological recognition event into a signal (mass, charge, heat or light) measurable - a process that is known as pointing. Most transducers produce light or electrical signals that are generally proportional to the concentration of the analyte. While the processing unit acts as a detector that filters, amplifies and analyzes the obtained signal (by the transducer). This unit consists of complex electronic circuits that convert an analogic signal into a digital signal and then transmit it to a screen or store it in a computer [47].

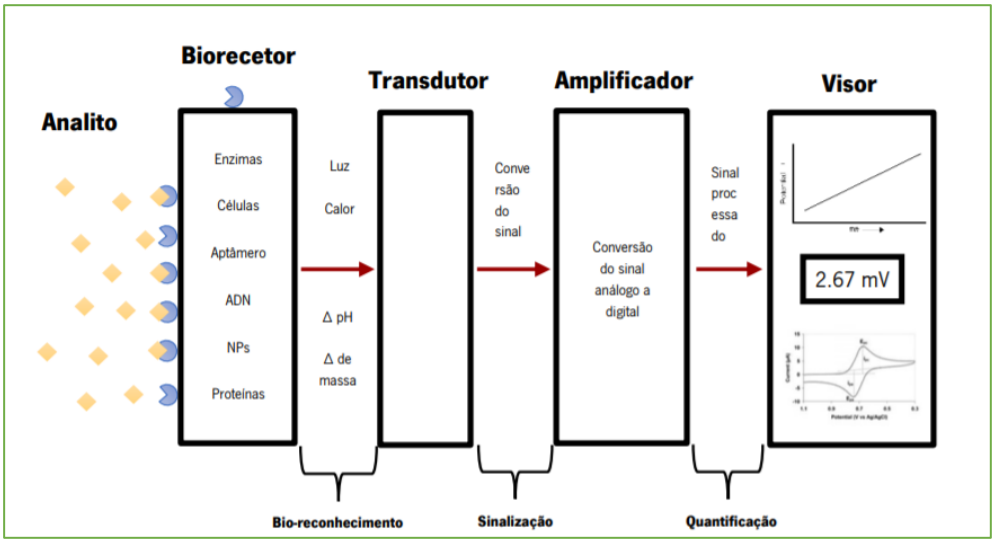

Figure 3- Schematic representation of a biosensor [49].

1.2.2. Biosensor History

The concept of a biosensor is not new. The first commercial biosensor was developed in 1975 by Yellow Spring Instruments. However, before their construction, the principles behind these devices were proposed and tested. These basics take us back to 1906 when M. Cremer showed that the concentration of acid in a liquid is proportional to the electric potential that arises between parts of the liquid on opposite sides of the glass membrane. However, the first "true" biosensor was only developed in 1956 by L.C. Clark, Jr.; for monitoring oxygen in blood [48]. Since that date and with the development of new technologies, many sensors have been built and improved for different purposes, providing better means for clinical diagnosis, not only for specificity in event detection but also for its rapid translation of signal, these devices have greater diversity and sensitivity in translating events and signals [49]. Biosensors have been studied for years as an alternative to traditional methods (e.g., fluorescence microscopy, FISH, spectroscopy, chromatography, PCR, molecular biology, etc...) [50].

1.2.3. Types Of Biosensors

Over the years, several types of biosensors have been developed, all with highly variable purposes, and three large groups can be considered depending on the transducer used: (1) optical, (2) electrochemical and (3) piezoelectric biosensors. Within these three groups of transducers, electrochemical is the most attractive for clinical observation and diagnosis, due to their low/moderate cost, simplicity, stability, high sensitivity and portability [51].

1.2.4. Biosensor Applications

Biosensors have a very wide range of applications aimed at improving the quality of life. This scope includes their use in environmental checking, disease detection, food safety, defense, drug discovery and many more. One of the major applications of biosensors is the discovery of biomolecules that are either disease indicators or drug targets. For example, electrochemical biosensing techniques can be used as clinical tools to discover cancer protein biomarkers [52][53]. Biosensors can also be used as platforms to monitor food traceability, quality, safety, and nutritional value [54][55]. Biosensors are used as technically advanced devices in both resource-limited and high-end medical settings: for example, with applications in drug discovery [56][57]; for the discovery of several chemical, and biological agents that maybe toxic

substances or of defensive importance [58]; for use in artificial implants such as defibrillators [59] and other prosthetic devices [60]. A range of electrochemical, optical and acoustic sensing techniques have been used, along with their incorporation into analytical devices for various applications. Figure 4 shows the different research areas where biosensors have been used.

Figure 4 - Major areas of applications for biosensors [46].

1.3. Development Of an Electrochemical DNA-Based Biosensor

In recent years, the development of selective and highly sensitive DNA-based biosensors has received increasing attention about efforts directed at gene analysis, genetic disorder and SNPs detection, tissue matching, forensic and more medical applications [61]. The electrochemical technology offers many advantages such as its simplicity, speed, low cost, and high sensitivity [62]. Various strategies have been devoted to the electrochemical detection of DNA hybridization. Electrophysiological indicators are commonly used as redox markers for the electrochemical detection of DNA hybridization [63]. In pursuit of the electrochemical detection of DNA with high sensitivity, nanostructures of different shapes, we were advised to modify the substrate to increase the amount of probe DNA immobilization and improve the recognition property of stable DNA [64]. Over the past several years, very high sensitivities have been demonstrated based on electrochemical assays based on nanomaterials. The introduction of nanomaterials can effectively increase the surface area of the electrode and expand the amount of DNA

immobilization. Furthermore, the nanostructure may play an important role in controlling the orientation and assembly density of the DNA probe for the ability to recognize optimal hybridization. Whereas these reported nanostructures usually participate in relatively complex preparation procedures, or the signal amplification effect of DNA electrochemical detection is limited. Thus, constructing a nanostructure-modified electrode with a simple strategy to achieve electrochemical detection of DNA with high sensitivity is highly desirable. The modified electrode of gold nanostructures can be easily constructed by a few different strategies including direct electrostatic assembly, covalent bonding, polymer retention or co-mixing, and electrodeposition methods. Among them, the electrodeposition method is one of the most widely used methods with the advantages of convenience and wide applications in electrical stimulation and electrolysis.

1.3.1. DNA Immobilization Onto Electrode Surface Strategies

The immobilization of a single-stranded DNA capture probe onto the electrode surface is the first step in the construction of a genosensor (biosensor able to detect the hybridization reaction between two complementary DNA strands) and the most important for its good performance. Depending on the type of transducer, the biomolecule under study and the application of the biosensor, the methods used for the DNA immobilization vary, also changing the conditions and experimental variables to be used [65]. The surfaces of sensor platforms (i.e., electrodes) may also vary in composition. As mentioned, the most common are carbon (namely graphite) and gold (Au) surfaces, which are usually modified with nanomaterials (carbon nanotubes) or Au nanoparticles (NP) to increase the selectivity and sensitivity of the methodology [66]. Some of the methods developed for immobilizing the probe on the electrode surface are (Table 1 and Figure 5) (i) adsorption (physical or chemical); (ii) affinity binding and (iii) covalent binding [66].

Table 1- Immobilization strategies for genosensors [67].

Strategies	***Binding nature***	***Advantages***	***Drawbacks***
Adsorption	Hydrophobic; Van der Waals or ionic interactions (weak bonds)	Simple, easy and cheap; Limits the loss of the enzymatic activity	Nonspecific adsorption; Desorption
Affinity	Affinity bonds between two partners (e.g. avidin/biotin)	Controlled and oriented immobilization; Great selectivity	Elevated cost; Requires specific functional groups
Covalent binding	Chemical binding between functional groups of the biomolecule and support	Stable; No diffusion barrier required; Short time response	Coupling with toxic product; Matrix and biomolecule cannot be regenerated
Entrapment	Incorporation of biomolecules in a gel or polymeric system	Wide applicability: Several types of enzymes may be immobilized simultaneously	Requires a diffusion barrier; Produces enzyme leakage; Needs a high concentration of monomers and enzymes

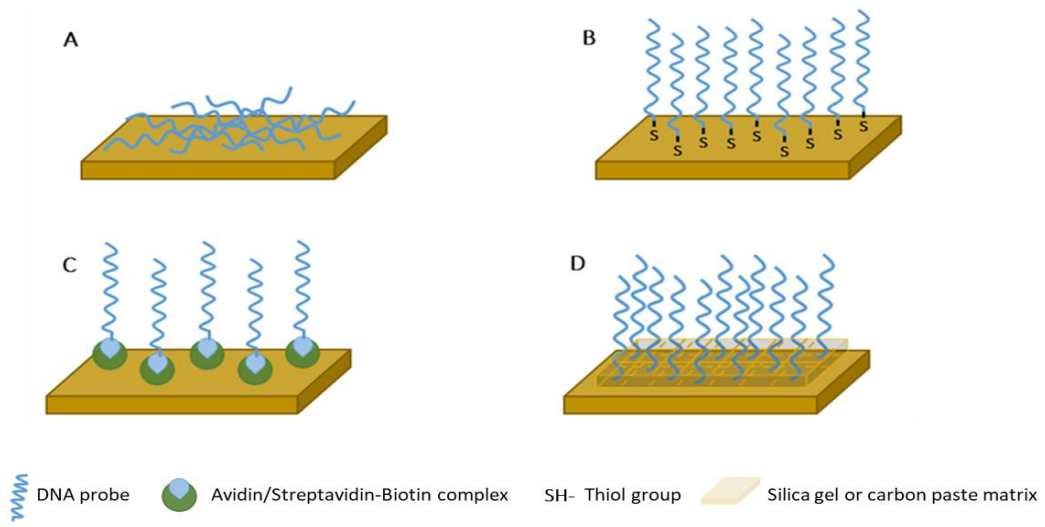

Figure 5 - Immobilization strategies. (A) Physical absorption; (B) Covalent bonding; (C) Affinity interaction; and (D) Matrix entrapment.

1.3.1.1. Physical Absorption

Physical adsorption is the simplest immobilization technique, as well as the weakest. Since this approach takes advantage of the intermolecular forces (such as the Van der Waal's, electrostatic, hydrophobic, and/or polar interactions) to attach the biological element onto the electrode's surface, it does not require

any chemical reagents or previous probe modifications [40]. However, this method is solely governed by the physical attractive forces between the biomolecule and the sensor's surface. For DNA detection, the ssDNA probes are immobilized on a sensor's surface thanks to the electrostatic adsorption between the negatively charged phosphate groups of the ssDNA probes on the positive charged electrode. Therefore, the number of adsorbed probes, their disposition and orientation along the surface area cannot be controlled. As a result, the ssDNA probes monolayer, as well as the probes that where not adsorbed on the surface are at risk of desorption [40].

1.3.1.2. Affinity Interactions

Affinity interactions are an excellent strategy to obtain the correct orientation of the biomaterials. There are several methods to create affinity bonds between a specific biomolecule and the electrodes surface, though the most reported technique is the formation of the avidin/streptavidin-biotin (avidin-biotin or streptavidin-biotin) complex. This results from the fact that biotin (a small molecule) binds, with a high affinity, to avidin's or streptavidin's binding sites (Ka = 1015 M^{-1}). Moreover, avidin/streptavidin are tetrameric proteins that provide four identical binding sites for biotin. Their tetrameric interactions can be exploited to immobilize DNA probes to an electrode's surface, by modifying the probes 3' or 5' end sequence with the biotin molecule and later adding it to the avidin/streptavidin-modified platform [68].

Additionally, the avidin/streptavidin-biotin complex is highly stable and resistant to extreme variations of temperature, pH, denatured detergents, and organic solvents [68].

Various methods for the functionalization of avidin/streptavidin on electrodes surfaces for immobilization of biotinylated DNA probes have been established for electrochemical genosensor detections. The most common strategy used is the EDC/NHC coupling reaction between activated carboxyl group and the avidin/streptavidin proteins [68].

Although the avidin/streptavidin proteins are easily immobilized on various types of electrodes surfaces their binding ability decreases over time. Furthermore, the synthesis of avidin/streptavidin-immobilized surfaces involves multiple steps, including the modification of the electrode's surface, the immobilization of proteins, among other. Each step increases the assay time and expense [69].

1.3.1.3. Covalent Bonds

The covalent immobilization approach involves chemical reactions that link the biomolecules to the electrodes surface by covalent bonds. Contrary to the absorption approach, covalent bonding demonstrates a good stability, flexible, highly binding strength and prevent desorption of the DNA probe's monolayer[68]. Moreover, covalent bonding provides a good vertical orientation where the end of DNA probe was grafted on the electrodes surface, exhibiting a high efficiency for the hybridization event and reduces the background noise coming from non-specific adsorption [68]. However, this technique usually requires a previous probe modifications with a functional group, namely the group of thiols (S-H) or amines (NH_2) at the end of 3' or 5' to guarantee the appropriate bind the specific functional group to the electrodes surface. This procedure led to high specific attachment of DNA probe onto the electrode surface and can prevent non-specific binding. Chemisorption and covalent attachment are frequently used in the covalent immobilization of DNA probe [68].

Chemisorption is often used to immobilize and form SAM of the thiol-modified DNA probes and the gold electrode surfaces, thanks to the strong affinity between the thiol group and gold surface, which forms a gold-sulfur (Au-S) covalent bonding [68]. Also, chemisorption of DNA probes AuNPs surfaces have gained popularity in recent years, due to their ability to increase the electrode's surface area[68]. In this case, any type of metal working electrodes is functionalized with AuNPs that act as the DNA probes immobilization site through the strong affinity interaction of the covalent Au-S bonds [68].

1.3.1.4. Matrix Entrapment

Entrapment is typically used when conducting polymers, such as silica gel and carbon paste, are employed as the three-dimensional (3D) matrix. In this case, the biomolecules of interest are incorporation in the electrode surface during the growth of the conducting polymer, which creates a more stable DNA surface. It is a fast and simple method, since it involves co-deposition of pyrrole and ss-DNA together in one step [70].

The silica gel entrapment methods involve the hydrolysis of alkoxide precursors under acidic (or alkaline) conditions, followed by the condensation of the hydroxylated units – leading to the formation of a porous gel, while the carbon paste entrapment incorporation biological components, allowing a faster electron transfer with versatile, stable and good reproducibility of the modified electrode [70].

1.3.2. Electrochemical Genosensors Design

Electrochemical genosensing, based on the use of sequence-specific oligonucleotides as a biorecognition element and the electrochemical transduction to convert the hybridization event into a detectable signal, has demonstrated great promise to address the deficiencies of the conventional nucleic acid analysis methodologies for the detection of circulating biomarkers associated with pathogenic, genetic, emerging and infectious diseases and inheritable and non- transmittable diseases, namely cancer and CVD [37].

Genosensors employ simple and relatively short chains of synthetic oligonucleotides as a biorecognition element, commonly referred to as probes [71]. Although genosensors exploit a range of distinct chemistries, the chemical affinity that results from the formation of the unique hydrogen bridges between each pair of nitrogen bases during the DNA hybridization (A with T and C with G) is the rule that dictates the robustness and specificity of genosensors [72]. These sensors take advantage of the nanoscale interactions (hybridizations, ligations, or conformational changes) between the target present in solution, the recognition layer, and the electrode surface, to detect the electrochemical signal without the use expensive analyzers [37].Thus, it is enough that the base sequence of the target DNA molecule is known to allow the design and synthesis of its complementary probe, which can be immobilized on the transducer's surface, which then captures the signal from the hybridization reaction and transforms it into a measurable signal[68] .

Even though the specificity of genosensors has been demonstrated numerous times at its highest level (ability to discriminate sequences that differ on a single basis), their sensitivity is a parameter in constant improvement [73]. The detection of a specific DNA sequence present in the genomic material extracted from biological samples still requires, in most cases, a previous stage of amplification of this sequence by PCR. Even with maximum levels of sensitivity achieved (currently obtained with the use of nanomaterials), the influence of the background noise and other external interfering factors (e.g., temperature, pH, light) from the analyzed sample or from the analysis environment on the generated signal is another challenging point in the development of genosensors]. The prospect, however, is that in the near future, genosensing technology will evolve enough to allow the detection of specific DNA sequences directly from biological fluids, without any prior purification or amplification process [37].

As a general example, Figure 6 portraits a schematic display of the (three) main steps involved in the electrochemical detection of a genosensors for a particular DNA target using a sandwich hybridization format and the possible electrochemical detections methods.

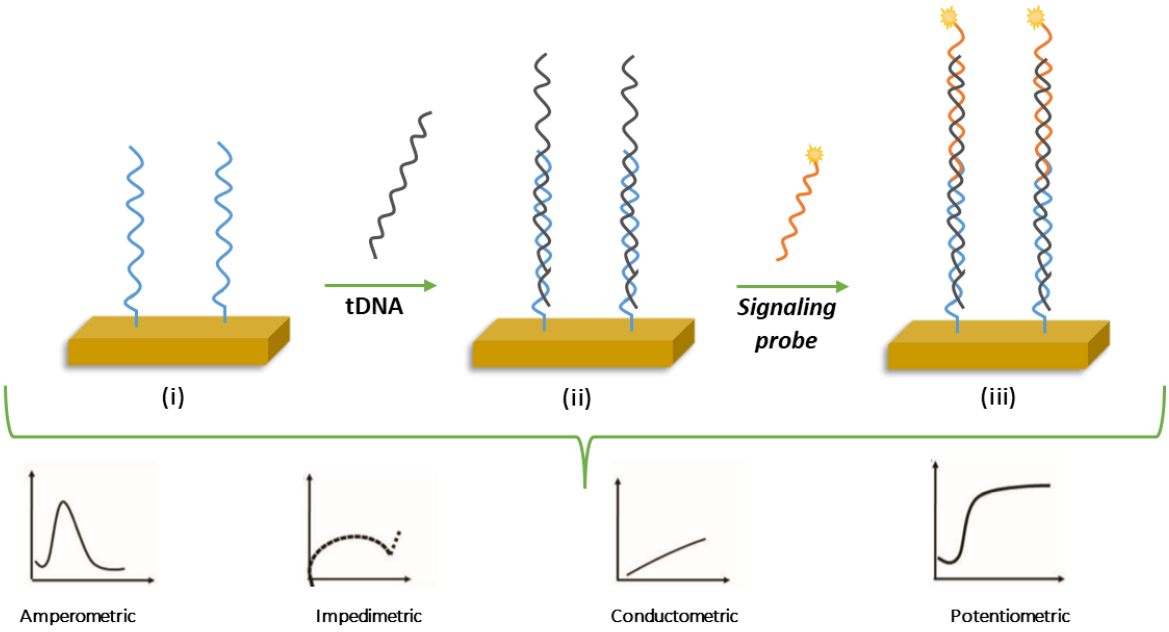

Figure 6 - Schematic display of a general genosensor for the detection of a particular nucleic acid sequence

involving (i) the immobilization of the specific capture probe on the electrode surface; and (ii) the hybridization reaction between the immobilized capture probe with the target sequence; and (iii) with the label signaling probe and the possible electrochemical signal detection methods.

2. Aim Of The Thesis

The main propose of this research work was to develop new fast selective and sensitive electrochemical genosensors for the CVD SNPs detection.

The specific aims include:

1- Development of an electrochemical genosensor for the VKORC1 SNPs gene (associated to CVDS) detection.

2- Using public data base (e.g., NBCI) select specific DNA sequence for the genosensor construction.

3- Construction and design the electrochemical genosensor.

4- Optimization, characterization and evaluation of the experimental variables associated to the electrochemical genosensor.

3. Materials and Methods

3.1. Chemical and Solution

All chemicals were of an analytical grade, so no further purification was required. 6-mercapto-1-hexanol (MCH), saline sodium phosphate 20 × (200 mM sodium phosphate, 3 M NaCl, 20 mM EDTA) pH 7.4 (20 × SSPE), 3,3 and 5,5' tetramethylbenzidine (TMB, Neogene K-blue enhanced activity substrate containing H_2O_2) were purchased from Sigma-Aldrich. Absolute ethanol and ethanol 96% were acquired from PanReac|AppliChem. Phosphate-buffered saline (PBS) and conjugated anti-fluorescein Peroxidase (anti-FITC-POD) was purchased from ThermoFisher and Roche Diagnostics, respectively.

The 20 × SSPE solution was diluted with ultrapure Milli-Q water (18.2 MΩ cm) from a Millipore purification system (20 ml of 20 × SSPE buffer in 180 ml of water) to prepare the 2 × SSPE buffer that was used as working buffer solution.

The construction of the electrochemical genosensor, depends on the specificity of the chosen VKORC1 SNP DNA probes. Hence, after analysing public SNP databases, two specific 52 bp target sequences; one with the adenine (TA) and another with the guanine (TG) SNP genetic variation were selected and designed. The oligonucleotide sequences (Table 2) used in this experiment were purchased from Sigma-Aldrich as a lyophilized salt. Every oligonucleotide stock solution (100 nM) was prepared with Milli-Q ultrapure water and stored at – 20 ºC, while the working oligonucleotides were prepared daily by diluting the desired concentration in the 2x SSPE buffer.

The target's complementary probes capture and signalling probes were divided as a 25 bp and 27 bp sequence, respectively (Figure 7). The DNA-capture probe was functionalized with a thiol group at the 5' end to enable its attachment onto the gold substrate, whereas the signalling probe was functionalized with a protein – fluorescein – at its 3' end. Theoretically, the capture and signalling probes will form a perfect and rigid duplex with the complementary target sequence.

Table 2 - Oligonucleotide sequences. Bold letters represent the single-nucleotide polymorphism (SNP) variation site.

Oligonucleotide	Sequence 5' → 3'	Bp
DNA-Capture probe	SHC_6OH–CTGAAAAACAACCATTGGCCTGGTG	25
DNA-Signaling probe	FC–CGGTGGCTCACGCCTATAATCCTAGCA	27
DNA Target A (T_A)	TGCTAGGATTATAGGCGTGAGCCACCGCACC**A**GGCCAATGGTTGTTTTTCAG	52
DNA Target G (T_G)	TGCTAGGATTATAGGCGTGAGCCACCGCACC**G**GGCCAATGGTTGTTTTTCAG	52

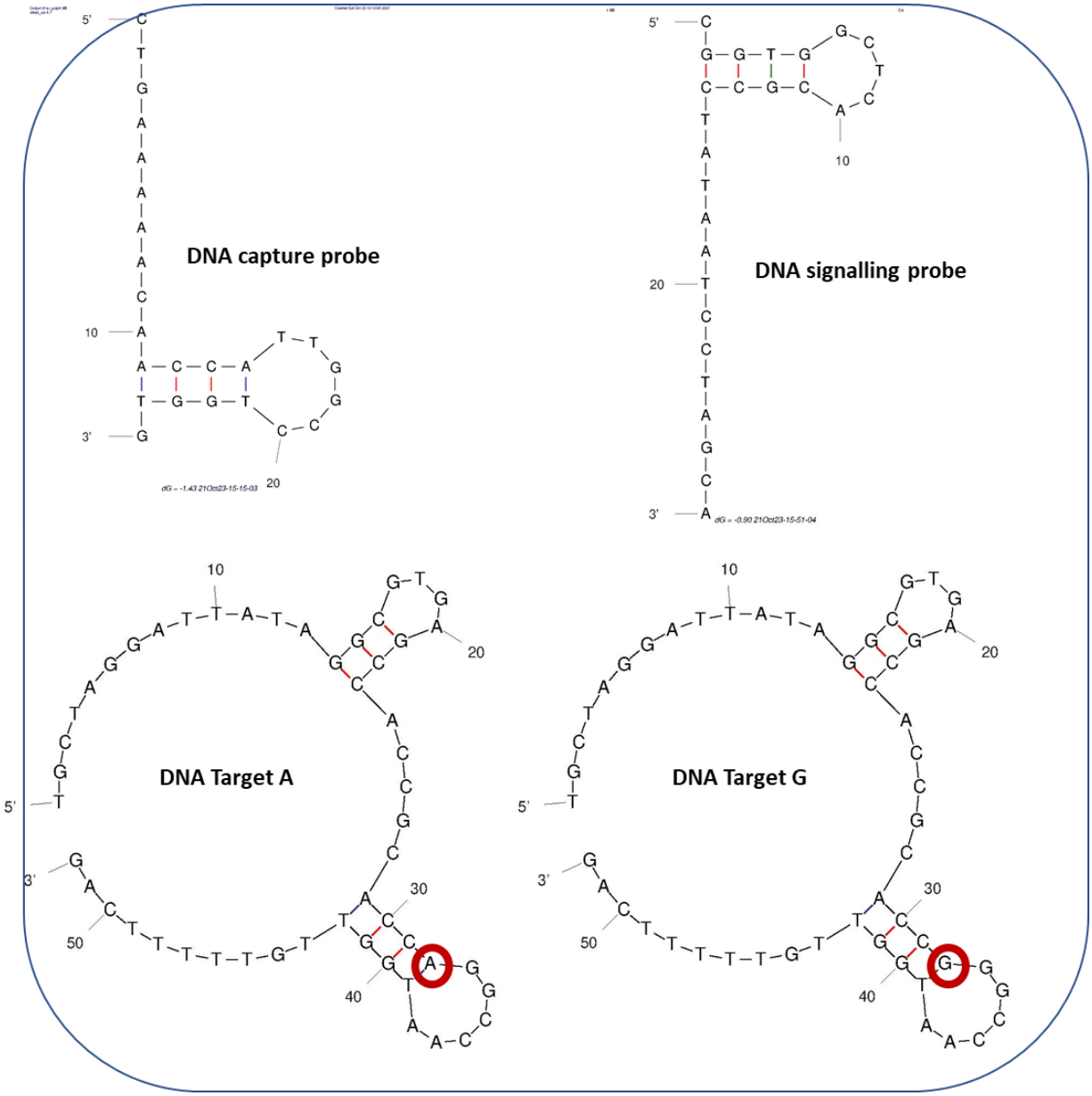

Figure 7 - DNA structures (data from www.ncbi.nlm.nih.gov/blast)

3.2.Device and Electrodes

Gold screen-printed gold electrodes (AuSPGE) obtained from Metrohm were used as the electrochemical transducer. The electrodes consisted of a gold working electrode (Ø 1.6 mm), a pseudo silver reference electrode and a counter electrode, L 33 × W 10 × H 0.5 mm. Electrochemical, namely chronoamperometric

measurements were executed on an AutoLab potentiostat (Metrohm) via NOVA 1.11.2 software. All experiments were accomplished at room temperature (25 ± 0.5 °C).

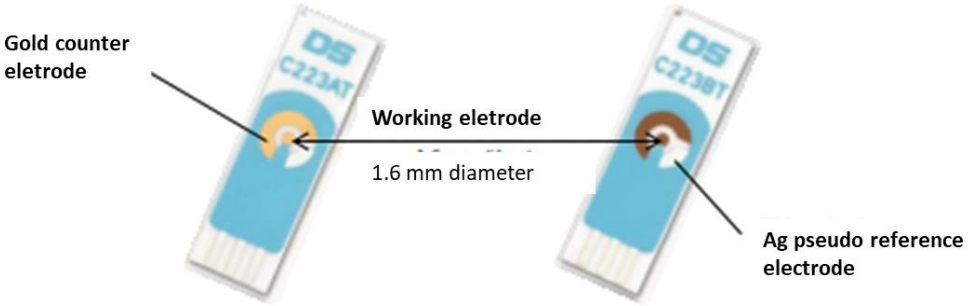

Figure 8 - Screen-printed gold electrode format.

3.3.Electrochemical Genosensor Construction Protocol

The genetic biosensor construction model includes, in short, four stages (Figure 9): i) pretreatment; ii) sensing phase, iii) sandwich DNA hybridization reaction and iv) electrochemical detection.

In the first stage the AuSPEs were cleaned with pure ethanol and ultrapure water and dried with nitrogen flow before use (pretreatment).

In the sensing phase in was performed the DNA capture probe and MCH immobilization. For that a self assembled monolayer (SAM) interface was carried out by assembling the linear DNA capture probe and MCH onto the working AuSPE surface. For that, 3 µl of 1 nM DNA capture probe was inserted onto the working electrode and stored in a humidified Petri dish overnight. Then, the modified SPGE was rinsed twice with 200 µl of 2 × SSPE buffer (to remove the weakly attached DNA probes) and 3 µl of MCH (1 µM) was added to the modified electrodes (sensing phase). The sandwich hibridization assay was obtained in a two-step hybridization. First, the homogenous hybridization occured when a DNA signaling probe (0.25 µM) binds to the DNA target in buffer solution, for 30 min. Then it was promoted the heterogenous hybridization by adding the result of the homogenous hybridization onto the modified electrode (DNA-AuSPEs). After 60 min the total hibridization was carried out. Then, the electrodes were rinsed with 2 x SSPE to remove any dense nonspecific DNA chains.

At the end and in order to amplify the electrochemical signal it was added to the modified dsDNA-AuSPEs an anti-fluorescein antibody conjugated with horseradish peroxidase (POD) enzyme. Enzymatic amplification of the electrochemical signal was obtained through chronoamperometry get through a POD/H_2O_2 system. Enzymatic labelling using monovalent bonds provides an improvement in terms of detection limits, while at the same time introducing scaling selectivity [65].

In the end, the genosensor was connected to a potentiate and 40 µl of TMB/H_2O_2 substrate was added to cover the electrode for 1 min (Figure 9). Detection of the of the enzymatically oxidized product was made by chronoamperometry at – 0.1 V, for 60 s.

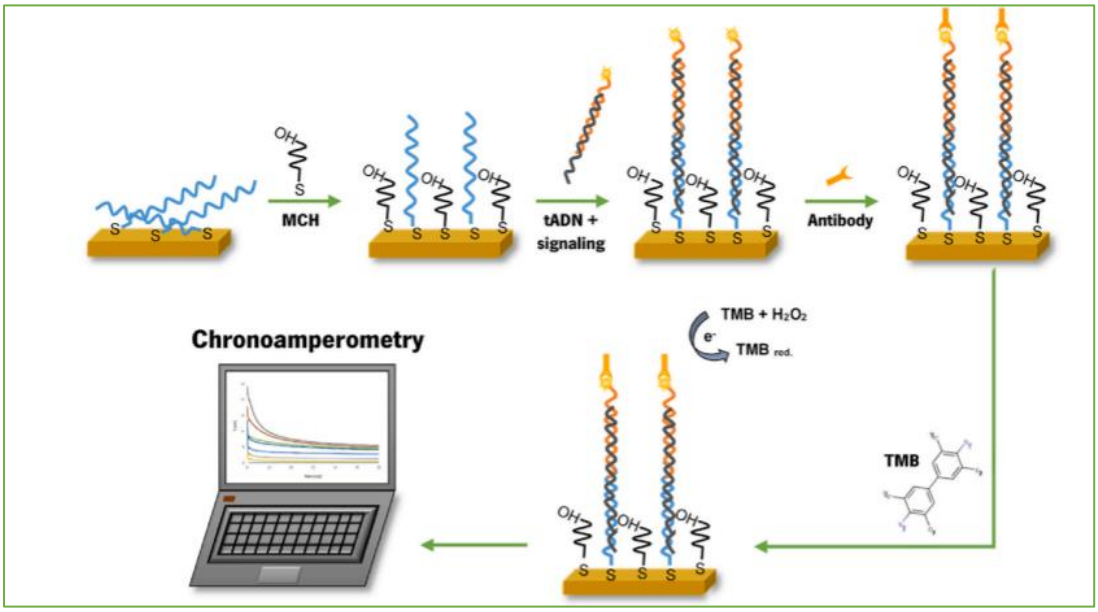

Figure 9 - General procedure for the development of an electrochemical Geno sensor [65].

4. Results and Discussion

4.1. Selection Of DNA Probes for The SNPs VKORC1 Genosensor

The construction of a genosensor for the SNPs VKORC1 detection requires the selection of a DNA oligonucleotide sequence specific of the VKORC1 gene.

In this work, for the construction of the SNP-specific electrochemical genosensor, two 52-mer oligonucleotide sequences; one with (T_A) and another with (T_G) VKORC1 gene variant, were selected (Table 2). This oligonucleotide has a secondary structure with a Gibbs energy of ΔG = − 5.09 kcal mol^{-1} and – 4.40 kcal mol^{-1} for DNA Target A and DNA target G, respectively, under the assay conditions (T = 25 °C and $[Na^+]$ = 0.298 mol L^{-1}) calculated using online tools [74] and suitable for genosensing. The capture and signaling probes were also designed to minimize secondary structures, while forming a perfect duplex structure after hybridization on the AuSPEs avoiding fringe regions that are deleterious for the analytical performance. The most stable DNA structures have a Gibbs energy of −1.43 kcal mol^{-1} and -1.89 kcal mol^{-1} for a 25 nt DNA capture probe and a 27 nt signaling probe, respectively. This ensures a facilitated surface hybridization. All sequences are shown in Table 1 and Figure 7.

4.2.Study of the SNPs Effect on the Electrochemical Genosensor

The principal objective of this work was the development of electrochemical genosensors able to identify and discriminate the electrochemical current between two SNP polymorphisms of the VKORC1 DNA sequences. To study the effect of SNP onto the electrochemical hybridization reaction, two different electrochemical genosensors were construction by using as DNA complementary sequences the DNA Target A and the DNA Target G. As it is possible to observe in Table 2 and Figure 7, the DNA Target A is totally complementary to the DNA capture probe and the DNA Target G presents one mismatch (at the 32 pb, the adenine was substituted by guanine), so it was only partially complementary. Figure 10 shows the chronoamperograms obtained during this study.

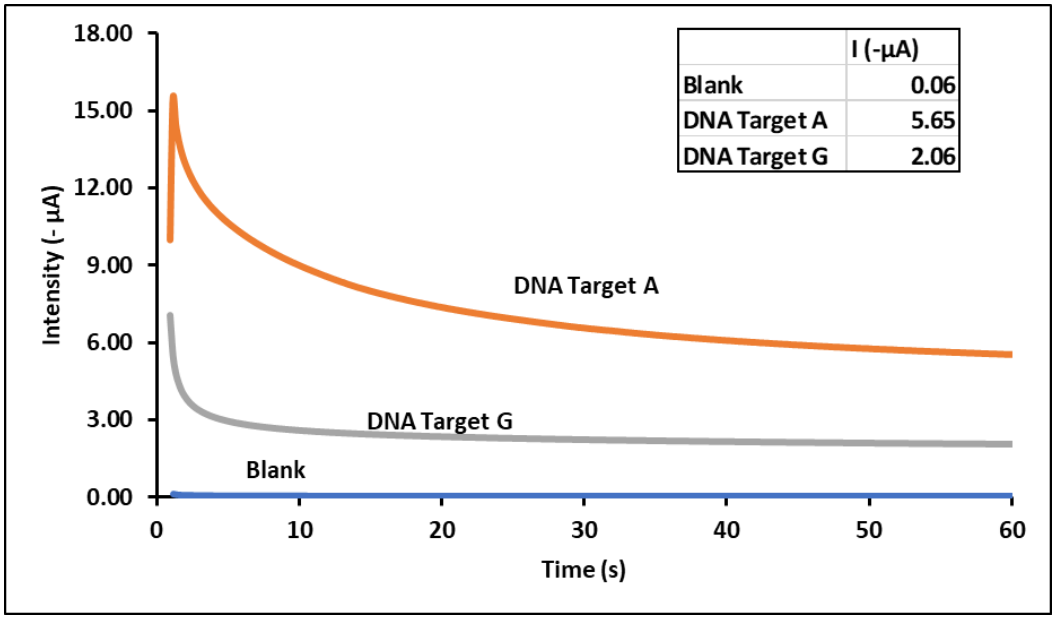

Figure 10 - Chronoamperograms obtained for the blank assay and when it was used the DNA Target A and DNA Target G as DNA complementary sequence.

Comparing the electrochemical current obtained when the DNA Target A was used as the total DNA complementary sequence (I= - 5.65 x10^{-6} A) to the electrochemical current when the DNA Target G was used (I = - 2.06x10^{-6} A), it was possible to observe that the electrochemical currents were higher (2.74 times higher) when the DNA sequences were totally hybridized (DNA Target A).

So, it is easy to conclude that the electrochemical genosensor was able to identify and discriminate the presence of a SNP in a DNA sequence.

4.3. Optimization Of the Experimental Variables

Most of the experimental parameters, namely the concentration of the DNA-capture, and two DNA-target probes, incubation time of the DNA-signaling probe, concentration and incubation time of the antibody and spacer involved in the genosensors development were optimized.

To determine the influence of the DNA-capture probe concentration in the intensity of the electrochemical currents, DNA-capture concentrations ranging from 0.50 to 2.00 µM were immobilized

on the working electrode surface. The electrodes with the highest S/B ratio value, for both the TA and TG DNA-target probes (S/B = 201 and 52, respectably), as well as the highest cathodic electrochemical current (Inc) were those immobilized with 1.00 µM of the DNA-capture probes (Figure 11). Henceforth, all optimizations will proceed using 1.00 µM of the DNA-capture probe.

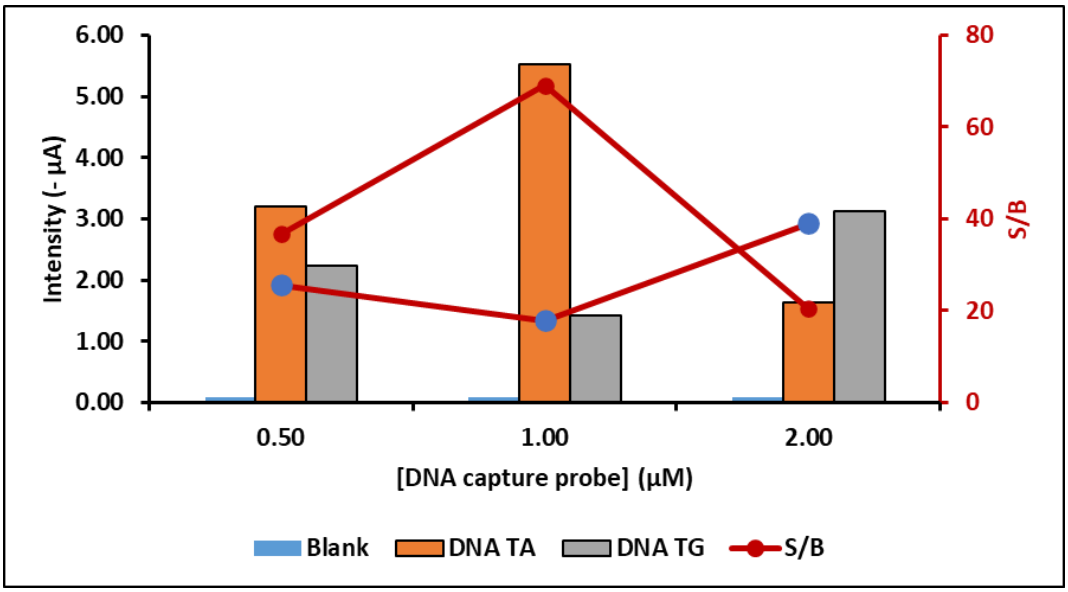

Figure 11 - Effect of the DNA capture probe concentration on the chronoamperometric response.

Blank (B) values represented in blue, signal (S) in orange for the DNA Target TA, in grey for the DNA Target TG, and corresponding S/B ratio in red.

The next step was the optimization of the concentration and incubation time of the SAM assembled onto SPGE. Under the same analytical conditions (1.00 µM of the DNA-capture probe and 0.50 nM of DNA target, at 25 ^{o}C), different concentrations of MCH ranging from 0.50 to 2.00 mM incubated over short periods of time, 15 to 45 min., were tested. The best Inc and S/B ratio values for the TA and TG probes were obtained when the AuSPE was immobilized with 1.00 µM (S/B 61.3 for TA and 15.9 for TG) (Figure 12). In what concerns the MCH incubation time, the best Inc and S/B ratio values for TA and TG probes were obtained when it was used and incubation time of 30 minutes (S/B = 9.14 and 5.36 for TA and TG respectively) (Figure 13). So, a MCH concentration of 1 mM and an MCH incubation time of 30 min was used for the next optimization steps.

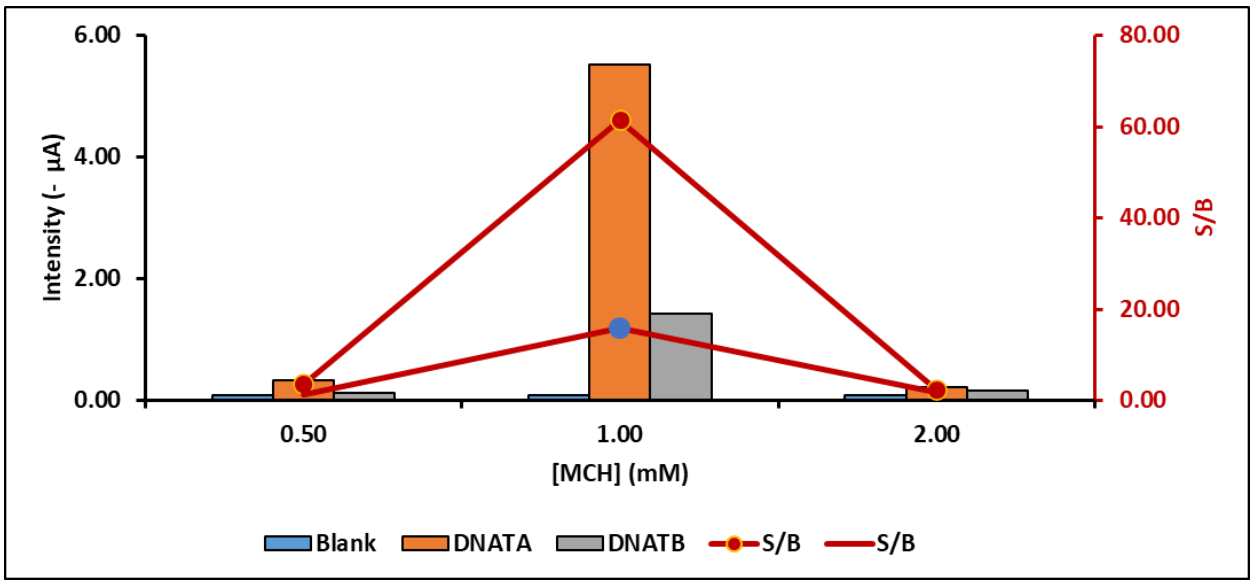

Figure 12 - Chronoamperometric response obtained when studying the influence of the concentration of MCH spacer.

Blank (B) values represented in blue, signal (S) in orange for the DNA Target TA, in grey for the DNA Target TG, and corresponding S/B ratio in red.

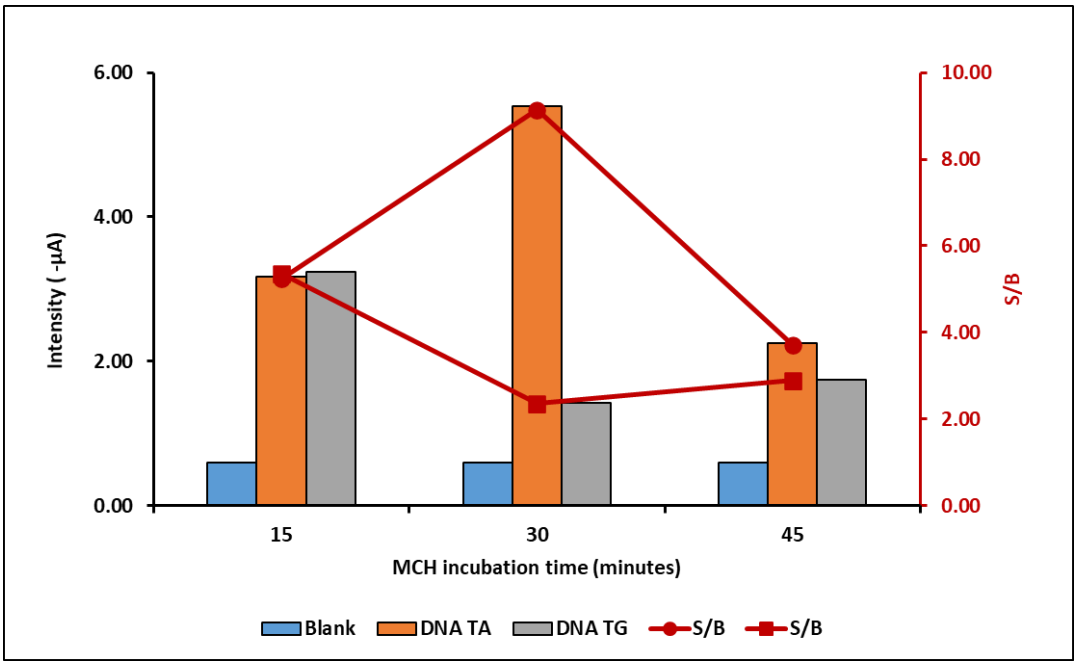

Figure 13 - Chronoamperometric response obtained when studying the influence of the incubation time of MCH spacer.

Blank (B) values represented in blue, signal (S) in orange for the DNA Target TA, in grey for the DNA Target TG, and corresponding S/B ratio in red.

The anti-FITC-POD enzyme is incorporated to the fluorescein protein label on the DNA duplex through an affinity interaction. So, when the TMB/H_2O_2 substrate is added for the chronoamperometric detection of the hybridization process, the amount of POD enzymes should be directly proportional to the number of hybridized sequences on the electrode's surface. In order to determine its influence in on the genosensors performance, several concentrations of antibody ranging from 0.50 to 2.0 U/mL were incubated on the genosensor, over an extended period of time: 15 to 45 min. Higher S/B ratios (as well as the highest Inc) were obtained when 1.0 U/mL of the anti-FITC-POD enzyme were applied to the electrode (S/B = 5.13 for TA and S/B = 4.32 for TC) (Figure 14).

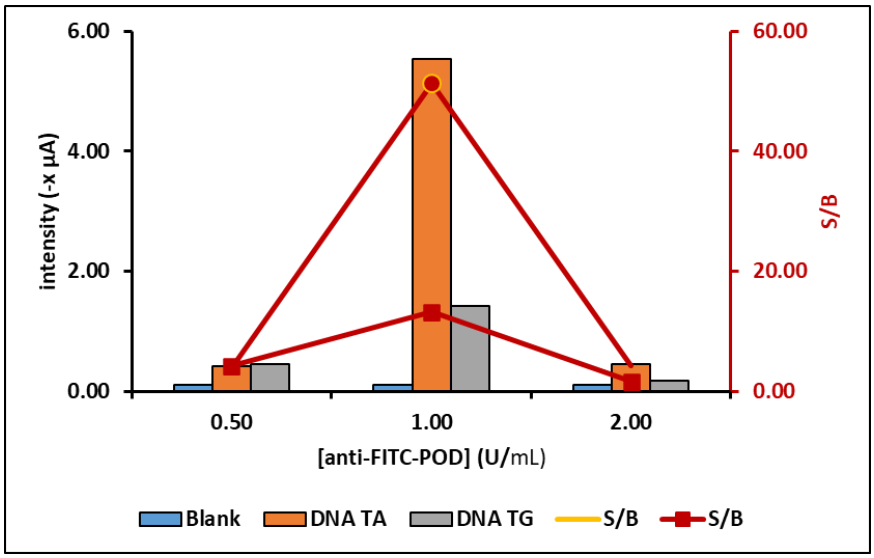

Figure 14 - Chronoamperometric responses acquired when optimizing the concentration (of the antibody Anti-FITC-POD.

Blank (B) values represented in blue, signal (S) in orange for the DNA Target TA, in grey for the DNA Target TG, and corresponding S/B ratio in red.

Regarding the anti-FITC-POD incubation time, the best S/B ratio for TA (S/B = 51.3) and TG (S/B = 19.1) was obtained when the affinity interaction between the antibody and the DNA duplex was held for 30 min (Figure 15). Table 3 summarizes all the selected experimental parameters as well as the test ranges in which they were optimized.

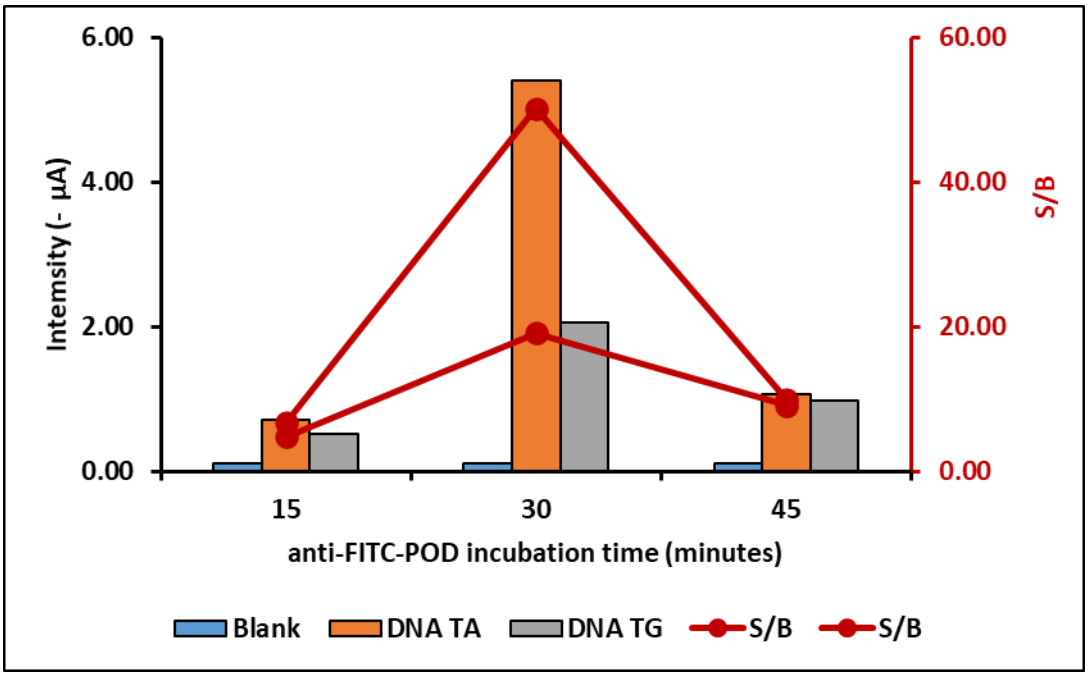

Figure 15 - Chronoamperometric responses acquired when optimizing the incubation time of the antibody Anti-FITC-POD.

Blank (B) values represented in blue, signal (S) in orange for the DNA Target TA, in grey for the DNA Target TG, and corresponding S/B ratio in red.

Table 3 - Selected values for the electrochemical genosensor construction.

Variables	Tested range	Selected value
DNA Capture probe concentration (µM)	0.50–2.00	1.00
MCH concentration (mM)	0.50–2.00	1.00
MCH incubation time (min)	15–45	30
Antibody concentration (U/mL)	0.50–2.00	1.00
Antibody incubation time (min)	15–45	30

4.4. Analytical Characteristics

Under the optimized experimental parameters (Table 3), the effect of increasing 52-mer synthetic DNA Target TA and DNA Target TG concentrations on the analytical signal was assessed by determining the chronoamperometric current from 0.050 to 1 nM (Figure 16 and 17).

A linear relationship (r^2 = 0.998) between the blank-subtracted intensity current (I_{net}) and the synthetic target concentration was obtained in the 0.050 to 1.00 nM range, with a slope and intercept value of

10.52 ± 0.14 (µA/nM) and 1.39 ± 0.07 (µA), for DNA Target A and DNA Target G, respectively (Figure 16a inset and 17a inset).

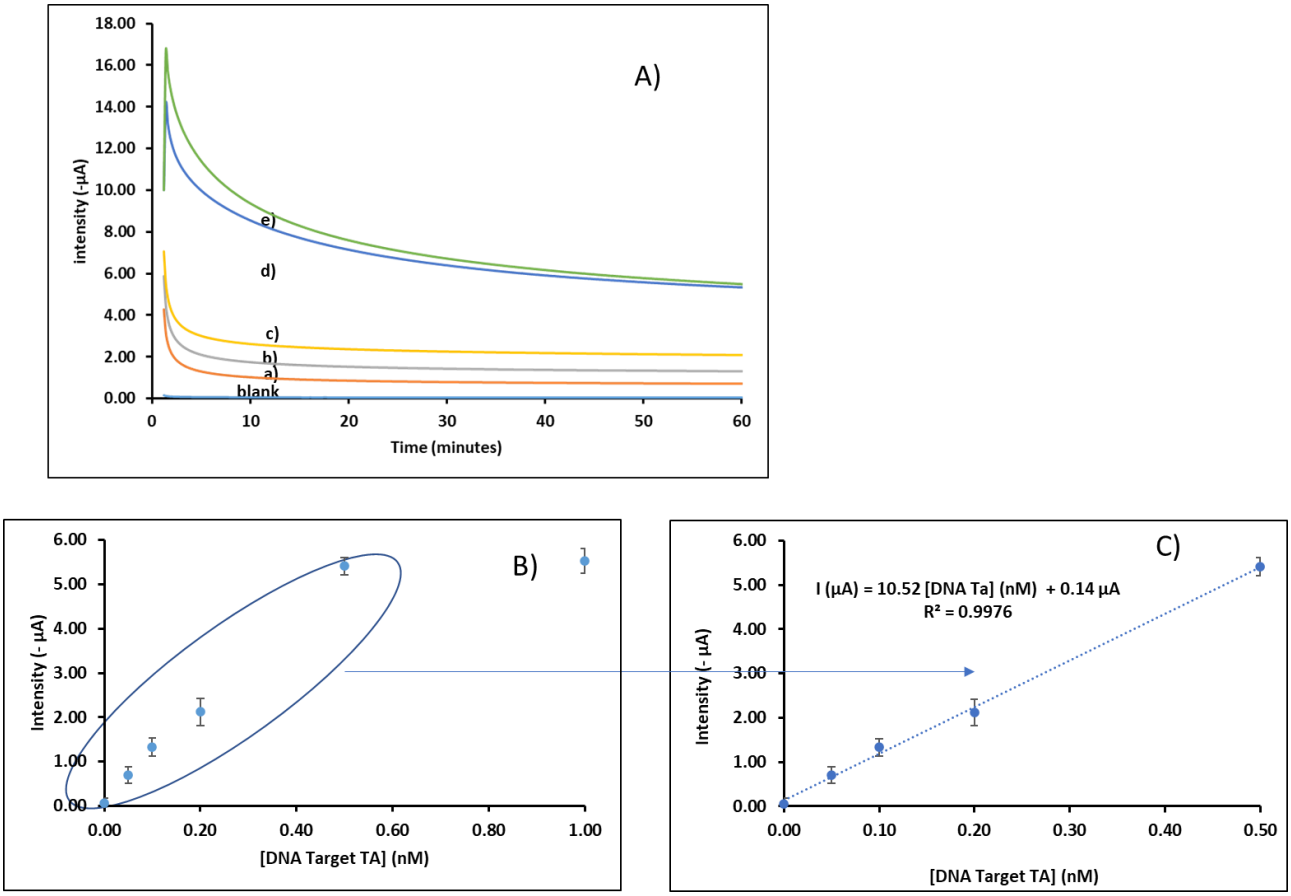

Figure 16 - A) Chronoamperometric responses for different DNA Target TA concentrations (a) 0.05; b) 0.10; c) 0.20; d) 0.50 and e) 1.00 nM). B) and C) Variation of the blank subtracted current with the DNA Target TA concentrations.

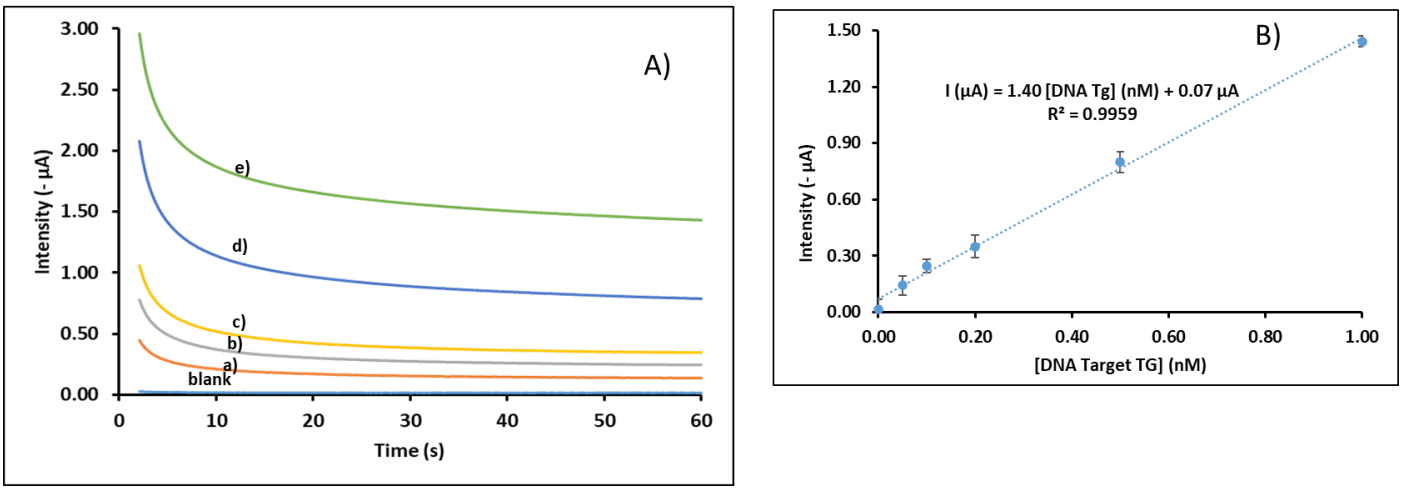

Figure 17 - A) Chronoamperometric responses for different DNA Target TG concentrations (a) 0.05; b) 0.10; c) 0.20; d) 0.50 and e) 1.00 nM). B) and C) Variation of the blank subtracted current with the DNA Target TG concentrations.

Comparing the TA/TG ratio (ranging from 3.0 to 7.0) acquired from the chronoamperometric measurements (Figure 18), it is easy to conclude that the developed electrochemical genosensor is able to identify and discriminate between the two polymorphic DNA sequences. Furthermore, higher target concentrations present a higher TA/TG ratio value.

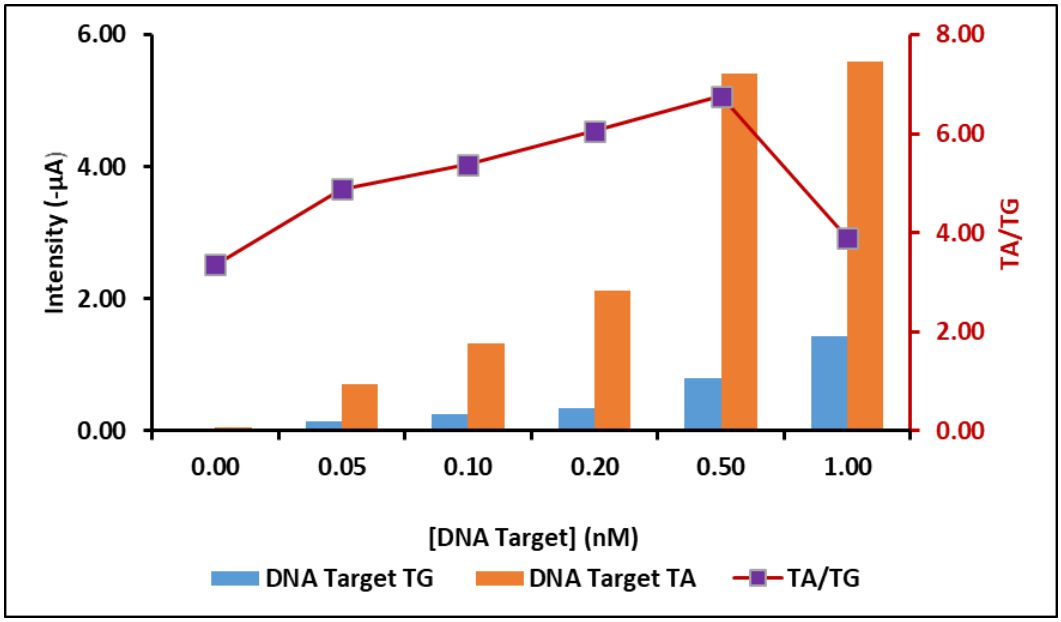

Figure 18 - Chronoamperometric responses obtained when studying the influence of increasing concentrations of the synthetic DNA target sequences.

Current values of the non-variant target probe (TA) represented in orange, altered target probe (TG) in blue and the corresponding TA/TG ratio in red.

The limit of detection (LOD) and the limit of quantification (LOQ) calculated as three times and ten times the standard deviation of the blank assay divided by the slope of the calibration plot were 20.0 pM and 90.3 pM, respectively (Table 4).

The precision of the genosensors were assessed by using 0.50 nM of DNA Target. For that, the repeatability was determined by inter-electrode measurements and the reproducibility was evaluated by carrying out three measurements in five consecutive days. Repeatability and reproducibility expressed as relative standard deviation were 6.549% and 5.41%, respectively.

Table 4- Analytical parameters of the developed electrochemical genosensors.

Parameters	**DNA Target TA**	**DNA Target TG**
Linearity (nM)	0.05 – 0.50	0.050 – 1.00
Slope (µA/nM)	10.52	1.39
Interception (µA)	0.41	0.07

Correlation (R)	0.9988	0.9959
Slope standard deviation (µA/nM)	0.06	0.04
Interception standard deviation (µA/nM)	0.03	0.005
LD (pM)	20.0	22.4
LQ (pM)	90.3	86.4
Repeatability	6.54	5.21
Reproducibility (%)	5.41	4.86

5. Conclusion

In this work a disposable SPGE capable of discriminating between the two VKORC1 genotypes was developed and optimized.

The genosensor's construction was based on the creation of a mixed SAM, on the gold surface, consisting of thiolate compounds – thiolate capture DNA (SH-DNA) and MCH. To increase the sensor's selectivity, the hybridization reaction was performed in a sandwich format, the amplification of the electrochemical signal was executed through the POD enzyme, and chronoamperometry was used to detect the electrical currents.

All optimized parameters (DNA Capture probe concentration, MCH time and concentration, and the antibody time and concentration) contributed to enhance the sensor's detection sensitivity and, consequently, reduce its detection limit.

The developed sensor displayed a good performance by discriminating between the two SNP target (TA and TG) sequences. The utility of this analytical device as an alternative to the conventional genotyping methodologies can easily unburden the public health system and, hopefully, prevent drug related CDV episodes, such as those caused by the excessive or inadequate dose of warfarin.

6. Future Prospective

Despite all the scientific work in this field and the results obtained, more research is still needed. As a continuation of this work developed within the scope of this master's thesis.

Suggestions:

- Validation of the electrochemical genosensor using, as reference methodology, molecular biology techniques such as the conventional PCR and the real time PCR;
- Compare the sensitivity of these developed electrochemical genosensor with traditional methodologies such as PCR;
- Application of the novel genosensor for the determination of the populational CYP2C9*3 genetic variations related with the therapeutic response of warfarin;
- Implementation of this genosensors on the cardiovascular field where pharmacogenetics is likely to play an important role in this field;
- Evaluation of the possibility of increasing the sensitivity of electrochemical biosensors using selected nanomaterials.

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