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Development of electrochemical genosensors applied to cardiovascular pharmacogenetics †

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Abstract: Cardiovascular diseases (CVD) are considered one of the leading causes of death worldwide. To prevent cardiovascular complications and further loss of life oral anticoagulants (e.g., warfarin) are frequently prescribed to patients. Nevertheless, warfarin therapeutic agent presents narrow therapeutic windows with well-documented health risks. Some of these dose-responses are a result of specific single-nucleotide polymorphism (SNP) genetic variations present in a patient's DNA. Among them, determined SNP in the cytochrome P4502C9 (CYP2C9), namely the CYP2C9*3, gene has been identified as dose-response altering SNP. Therefore, the need for a rapid, selective, low-cost and in real time detection device is crucial before prescribing any anticoagulant. This work addresses the development of a disposable electrochemical genosensor capable of detecting SNP in the CYP2C9*3 allele. Analyzing public databases, two specific 78 bp DNA probes; one with the adenine (TA) and another with the cytosine (TC) SNP genetic variation were selected and designed. The genosensor methodology implied the immobilization of a mixed self-assembled monolayer (SAM) linear CYP2C9*3 DNA-capture probe and mercaptohexanol (MCH) onto screen-printed gold electrodes (SPGE). To improve the genosensor's selectivity and avoid strong secondary structures, that could hinder the hybridization efficiency, a sandwich format of the CYP2C9*3 allele was designed using a complementary fluorescein isothiocyanate-labelled signaling DNA probe and enzymatic amplification of the electrochemical signal. Chronoamperometry measurements were carried out obtaining a concentration range from 0.015 to 1.0 nM for both TA and TC SNP target probes. Analyzing the results, the developed genosensor was capable of discriminating between the two SNP probes.

Keywords: Cardiovascular diseases; Chronoamperometry; Electrochemical genosensor; Sandwich format hybridization; Single-nucleotide polymorphism.

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1. Introduction

The human genome has been well conserved throughout our evolution, although, every now and then, it is slightly altered due to genetic variants known as mutations [1,2]. However, some mutations can cause crucial changes in ones' DNA, as permanent changes to the human DNA sequence can result in the variation or loss of function of a genetic product.

A variant in the DNA sequence that occurs with a frequency of 1% or higher in a population is designated polymorphism [2]. A single base-pair polymorphism is generally referred to as single nucleotide polymorphisms (SNP).

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 [3]. 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.

As one of the lead causes of death worldwide, cardiovascular diseases (CVD) are a major concern for every country, as well as a financial burden for the health care system. To combat CVD oral anticoagulants, such as warfarin, are prescribed as prevention methods [4].

Warfarin (vitamin K antagonist) is indicated for prevention of thromboembolic disorders associated with venous thromboembolisms, myocardial infarction, and strokes. However, despite its benefits, warfarin presents a narrow therapeutic window with well-documented risks in patients (e.g., hemorrhages or thromboembolisms). Around 66% of warfarin dose-responses variations are due to natural factors (e.g., age, body mass, gender, diet), while the remaining percentage can be caused by the interindividual genetic variations which produce variability in the dosage requirements [5].

The CYP2C9 gene has been identified as the main genetic determinant to warfarin's sensitivity. CYP2C9 activity is modulated by the presence of SNP, namely the CYP2C*3 allele was associated with the reduction of the enzymatic activity of CYP2C9. Therefore, patients with this SNP have an effective therapeutic response with lower doses [6].

Conventional methodologies, such as polymerase chain reaction (PCR) and real-time PCR, are the gold standard for genotyping studies. However, although sensible and specific, they are expensive, time consuming and require qualified professionals which makes these tests not a priority for population genotyping. Thus, it is imperative to develop new cost-effect genotyping technologies to implement pharmacogenetic analysis in public health systems [7]. A promising alternative are the electrochemical genosensors. Genosensors are analytical devices capable of detecting the hybridization reaction between two complementary DNA strands immobilized on a solid support [8]. Moreover, biosensors are used in point-of-care devices since they are portable, simple, easy to use and cost-effective. Despite the pointed advantages, no biosensors have been developed for this aim, making this an important field to explore.

This work reports the development of a disposable electrochemical genosensor with the capacity of distinguishing SNP between two 78 bp synthetic DNA sequences.

The genosensor's design implied the immobilization of a 25-mer DNA-capture onto disposable screen-printed gold electrodes (SPGE). To improve its selectivity and avoid strong secondary structures, that could hinder the hybridization efficiency, a sandwich hybridization format of the normal (TA) and variant (TC) CYP2C9*3 genes were designed using a fluorescein isothiocyanate (FITC) labeled signaling DNA probe to which anti-fluorescein antibodies labelled with horseradish peroxidase (POD) enzymes were attached. The enzymatic amplification of the analytical signal was obtained by chronoamperometry using a POD/H2O2 system.

The developed sensor displayed a good performance by discriminating between the two target sequences with the SNP. 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.

2. Material and methods

2.1. Apparatus and electrodes

Screen-printed gold electrodes (SPGE) (C223BT, DropSpen) purchased from Metrohm were utilized as the electrochemical transductor. These electrodes are composed

of a gold working electrode (Ø 1.6 mm), a silver pseudo-reference electrode and an auxiliary gold electrode on a flat ceramic chip.

All electrochemical measurements were performed on an AutoLab potentiostat (ΩMetrohm) by the NOVA 1.11.2. software. All experiments were carried out at room temperature (25 ± 0.5 ºC).

2.2. Reagents, samples and solutions

All the reagents were of analytical grade, so no further purifications were needed.

6-mercapto-1-hexanol (MCH), 20x sodium phosphate-EDTA (200 mM sodium phosphate, 3 M NaCl, 20 mM EDTA) pH 7.4 solution (20x SSPE) and 3,3′,5,5′ tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS), absolute ethanol and the anti-fluorescein-peroxidase (anti-FITC-POD) fragments were obtained from ThermoFisher, PanReac|AppliChem and Roche, respectively.

The 20x SSPE was diluted with Milli-Q ultrapure water (18.2 MΩ.cm) from a Millipore purification system in a 1:9 ratio to prepare the 2x SSPE buffer.

The results of this assay, as well as the construction of the electrochemical genosensor, depends on the specificity of the chosen SNP DNA probes. Hence, after analyzing public SNP databases, two specific 78 bp target sequences; one with the adenine (TA) and another with the cytosine (TC) SNP genetic variation were selected and designed. The oligonucleotide sequences (Table 1) 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 signaling probes were divided as a 25 bp and 53 bp sequence, respectably. The DNA-capture probe was functionalized with a thiol group at the 5′ end to enable its attachment onto the gold substrate, whereas the signaling probe was functionalized with a protein – fluorescein – at its 3′ end. Theoretically, the capture and signaling probes will form a perfect and rigid duplex with the complementary target sequence.

2.3. Electrochemical genosensor design

The developed genosensor design involves, essentially, four steps: a pretreatment, a sensing phase, the sandwich format hybridization, and the electrochemical detection.

Initially, all SPGE were washed with absolute ethanol and Milli-Q ultrapure water and dried with a nitrogen flow – Pretreatment.

To guarantee the probes orientation, a SAM interface comprised of linear DNA-capture probes and MCH was arranged. Therefore, 3 μL of the linear DNA-capture probe solution (1 μM) was immobilized onto the SPGE's working electrode and stored in a humified Petri dish for 24 hours.

The next day, the modified genosensor was rinsed twice with SSPE 2x buffer, in order to remove any weakly attached probes. Then, 3 μL of MCH (1 mM) was applied, for 30 min, to the working electrode – Sensing phase.

Afterwards, a sandwich format assay in a two-step (homogeneous and heterogeneous) hybridization was established. The homogeneous hybridization occurs when the DNA-signaling probe (0.125 μM) binds to the DNA target, for 30 min, in the buffer solution. The resulting solution was applied to the modified electrode, binding the target/signaling probe solution to the previously immobilized DNA-capture probes, for one hour – Sandwich format hybridization. All electrodes were then rinsed to remove any nonspecific adsorbed sequences.

The signaling probe was labeled with a fluorescein protein to which an anti-fluorescein antibody labelled with a horseradish enzyme is attached. To detect the electrochemical signal, 1.5 U/mL of POD enzymes in a PBS buffer solution were added to the electrode's surface, for 30 min, before being rinsed.

In the end, the genosensor was connected to the potentiostat and 40 μL of the TMB/H2O2 substrate was applied onto the electrode for 1 min. The detection of the enzymatically oxidized product was performed by chronoamperometry at – 0.1 V, for 60 s. Three replicates were used for all measurements.

Table 1. - Oligonucleotide sequences. Bold letters represent the single-nucleotide polymorphism (SNP) variation site.

Oligonucleotide	Sequence 5′ → 3′	Bp
DNA-Capture	SHC_6OH–GCTGGTGGGAGAAGGTAA**T**GTAT	25
DNA-Signaling	FC–CTCTGGACCTCGTGCACCACAGCATCTGTGTAGGG-CATGTGGCTCCTGTCTTG	53
Target A (T_A)	CAAGGAGCCACATGCCCTACACAGATGCTGTGGTGCAC-GAAGA-GAGGTCCAGAGAGATAC**A**TTGACCTTCTCCCCACCAGC	78
Target C (Tc)	CAAGGAGCCACATGCCCTACACAGATGCTGTGGTGCAC-GAAGA-GAGGTCCAGAGAGATAC**C**TTGACCTTCTCCCCACCAGC	78

SHC_6OH – thiol group; **FC** – fluorescein.

3. Results and discussion

3.1. Selection of DNA probes for sandwich format assay

For the construction of the SNP-specific electrochemical genosensor, two 78-mer oligonucleotide sequences; one with (TC) and another without (TA) the CYP2C9*3 gene variant, were selected.

The complementary sequence (to the TA target probe) was divided in two smaller DNA fragments: a 25 bp DNA-capture probe and a 53 bp DNA-signaling probe. Both probes were also designed to minimize the formation of secondary structures, seeing that on planar surface, such as the SPGE, strong secondary structures may hinder the hybridization process.

3.2. Optimization of the experimental variables

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

The aim of this study was to create an analytical platform capable of differentiating the three CYP2C9*3 genotypes, therefore in all of the experiments both the TA and TC DNA-target probes were utilized.

To determine the influence of the DNA-capture probe concentration in the intensity of the electrochemical currents, DNA-capture concentrations ranging from 0.25 to 1.00 μM were immobilized on the working electrode surface. The electrodes with the highest S/B ratio value, for both the TA and TC DNA-target probes (S/B = 170 and 101, respectably), as well as the highest cathodic electrochemical current (Inc) were those immobilized with 1.00 μM of the DNA-capture probes. Henceforth, all optimizations will proceed using 1.00 μM of the DNA-capture probe.

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 1.00 nM of DNA target, at 25 ºC), different concentrations of MCH ranging from 0.25 to 1.00 mM incubated over short periods of time, 7.5 to 30 min., were tested. The best Inc and S/B ratio values for the TA and TC probes were obtained when the SPGE was immobilized with 1.00 μM (S/B = 127 for TA and 46,7 for TC) and 0.25 mM (S/B = 124 and 46,9 for TA and TC respectably) of MCH, during 15 min. Nevertheless, the electrode with the third-best S/B ratio value (S/B = 114 for the TA and S/B = 38,8 for TC),

obtained when applied a MCH concentration of 1.00 mM for 7.5 min, was selected as the optimal value because it was the MCH concentration which presented the best TA/TC ratio value (2.94).

As previously mentioned, a sandwich hybridization format was adopted for this study. This format strategy was used because the two independent hybridization events (the homogeneous and heterogenous hybridization) that occur increase the overall selectivity of the assay [9]. Therefore, the incubation time for both hybridization steps were also optimized.

In this study, the homogeneous hybridization results from the partial hybridization between the DNA target and DNA-signaling probes, while the heterogeneous hybridization occurs between the target/signaling hybrid and the DNA-capture probes immobilized on the working electrodes surface.

For the homogeneous hybridization, the best S/B ratio for TA (S/B = 196) and TC (S/B = 102), was obtained after 30 min of incubation. As for the heterogeneous hybridization reaction the best S/B ratio values (S/B = 196 for TA and S/B = 100 for TC) was obtained after 60 min of incubation.

In order to determine the effect of the DNA-signaling probe concentration on the electrochemical responses, increasing concentrations of DNA-signaling from 0.123 to 0.50 μM were studied. The best S/B ratio (as well as the highest Inc) was obtained when 0.25 μM of the DNA-signaling probes was utilized. Future optimizations were performed with 0.25 μM of DNA-signaling probe.

The anti-FITC-POD enzyme is incorporated to the fluorescein protein label on the DNA duplex through an affinity interaction. So, when the TMB/H2O2 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 0.5 to 2.5 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 = 82.91 for TA and S/B = 35.40 for TC).

In regard to the anti-FITC-POD incubation time, the best S/B ratio for TA (S/B = 61.61) and TC (S/B = 36,61) was obtained when the affinity interaction between the antibody and the DNA duplex was held for 15 min. Table 2 summarizes all the selected experimental parameters as well as the test ranges in which they were optimized.

Table 2. - Selected values for the electrochemical genosensor construction.

Variables	Tested range	Selected value
DNA Capture probe concentration (μM)	0.25–1.00	1.00
MCH concentration (mM)	0.25–1.00	1.00
MCH incubation time (min)	7.5–30	7.5
Homogeneous hybridization incubation time (min)	15–45	30
DNA-signaling concentration probe (μM)	0.13–0.50	0.13
Heterogeneous hybridization incubation time (min)	30–120	60
Antibody concentration (U/mL)	0.50–2.50	1.00
Antibody incubation time (min)	15–45	15

3.3. Analytical characteristics

Under the selected experimental parameters (Table 2), the developed genosensor's analytical performance was assessed by chronoamperometry using increasing concentrations (0.015 to 5.00 nM) of the 78-mer synthetic DNA target probes TA and TC. A linear relationship (r2 = 0.9997) between the blank-subtracted intensity current (Inet) and the synthetic target concentration was obtained in the 0.015 to 1.00 nM range, with a slope and intercept value of 0,0095 ± 0.0001 (μA/nM) and 0.9055 ± 0.002 (μA), respectively.

Comparing the TA/TC ratio acquired from the chronoamperometric measurements (Fig.1), it is easy to conclude that the developed electrochemical genosensor is able to identify and discriminate between the two polymorphic DNA sequences. Furthermore, lower target concentrations present a higher TA/TC ratio value.

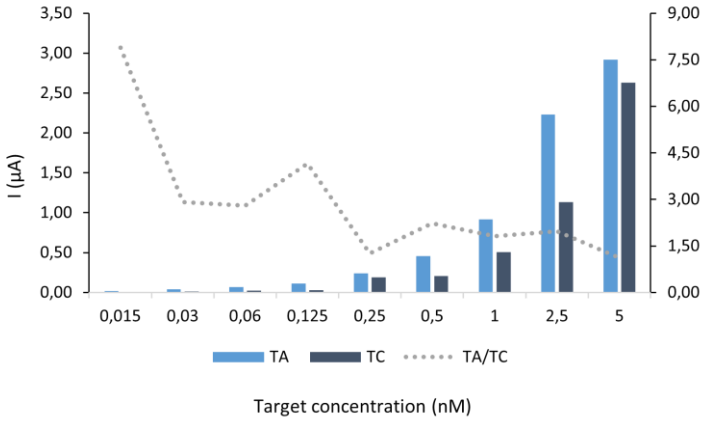

Figure 1. – Chronoamperometric responses obtained when studying the influence of increasing concentrations of the synthetic DNA target sequences. Current values of the non-variant target probe (T_A) represented in light blue, altered target probe (T_C) in dark blue and orange and the corresponding T_A/T_C ratio in grey. Analytical parameters: concentration of DNA-capture probe = 1.0 µM; concentration and incubation time of MCH = 1.0 mM, during 7.5 min; homogeneous hybridization incubation time = 30 min; heterogeneous hybridization incubation time = 60 min; concentration and incubation time of the DNA-signaling probe = 0.13 µM, during 15 min; and concentration and incubation time of the anti-FITC-POD antibody = 1.0 U/mL, for 15 min. Error bars estimate the standard deviation of three replicates.

4. Conclusions

A disposable electrochemical genosensor capable of detecting the synthetic CYP2C9*3 polymorphic DNA probes, with high selectivity and sensibility and in various concentrations, was developed.

All optimizations contributed to enhance the sensor's sensitivity. As a result, the developed electrochemical genosensor could discriminate between the two SNP target probes up to a concentration of 15 pM.

The genosensor's sensibility was attained by creating the mixed thiol capture DNA and MCH SAM compound on the working electrodes' surface. On the other hand, to increase the sensor's selectivity, a sandwich hybridization format was adopted, the electrochemical signal amplification conducted by the POD enzyme and chronoamperometry was utilized to measure the electrical currents.

Comparing the TA/TC ratio higher discriminating values are visible with lower SNP target concentrations. These results indicate that the developed electrochemical genosensor not only can identify an individual's CYP2C9*3 genotype, but this analytical device can be detected with low concentrations of DNA.

References

1. Altintas, Z.; Tothill, I.E. DNA-based Biosensor Platforms for the Detection of TP53 Mutation. Sensors and Actuators B. 2012, 169, 188-194.
2. Karki, R.; Pandya, D.; Elston, R.C.; Ferlini, C. Defining "mutation" and "polymorphism" in the era of personal genomics. BMC Medical Genomics. 2015, 8(37), 1-7.
3. Campuzano, S.; Yáñez-Sedeño, P.; Pingarrón, J.M. Electrochemical Genosensing of Circulating Biomarkers. Sensors. 2019, 17(4),1-20.
4. Nichols, M.; Townsend, N.; Scarborough, P.; Rayner, M. Cardiovascular disease in Europe 2014: epidemiological update. European Heart Journal. 2014, 35, 2929–2933.
5. Panchenko, E.; Kropacheva, E.; Dobrovolsky, A.; Titaeva, E.; Zemlyanskaya, O.; Trofimov, D.; Galkina, I.; Lifshits, G.; Vereina, N.; Sinitsin, S.; Vorobyeva, N.; Grehova, L.; Zateyshchikov, D.; Zotova, I.; Vavilova, T.; Sirotkina, O.; Grontkovskaya, A. CYP2C9

and VKORC1 genotyping for the quality of long-standing warfarin treatment in Russian patients. The Pharmacogenomics Journal, 2020.
6. Flora, D.R.; Rettie, A.E.; Brundage, R.C.; Tracy, T.S. CYP2C9 Genotype-Dependent Warfarin Pharmacokinetics: Impact of CYP2C9 Genotype on R- and S-Warfarin and Their Oxidative Metabolites. The Journal of Clinical Pharmacology. 2017, 57(3), 382–393.
7. Abad-Valle, P.; Fernández-Abedul, M.T.; Costa-García, A. DNA single-base mismatch study with an electrochemical enzymatic genosensor. Biosensors and Bioelectronics. 2007, 22, 1642–1650.
8. Sousa, J.B.; Ramos-Jesus, J.; Silva, L.C.; Pereira, C.; de-los-Santos-Álvarez, N.; Fonseca, R.A.S.; Miranda-Castro, R.; Delerue-Matos, C.; Santos Júnior, J.R.; Barroso, M.F. Fe3O4@Au nanoparticles-based magnetoplatform for the HMGA maize endogenous gene electrochemical genosensing. Talanta. 2020, 206, 120220.
9. Barroso, M.F.; Freitas, M.; Oliveira, M.B.P.P.; de-los-Santos-Álvarez, N.; LoboCastañón, M.J.; Delerue-Matos, C.; 3D-nanostructured Au electrodes for the event-specific detection of MON810 transgenic maize, Talanta. 2015, 134, 158–164.