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Detection of *Alexandrium minutum* dinoflagellate in environmental samples using electrochemical genosensor †

Stephanie L. Morais [1], Piedade Barros [2], Marlene Santos [2], Cristina Delerue-Matos [1], Andreia C. Gomes [3] and M. Fátima Barroso [1,*]

[1] REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto, Instituto Polit´ecnico, Rua Dr. Ant´ onio Bernardino de Almeida 431, 4200-072, Porto, Portugal
[2] CISA|ESS, Centro de Investigaçao ˜ em Saúde e Ambiente, Escola Superior de Saúde, Instituto Polit´ecnico do Porto, Rua Dr. Ant´ onio Bernardino de Almeida, 400, 4200- 072, Porto, Portugal
[3] Centro de Biologia Molecular e Ambiental (CBMA), Departamento de Biologia, Universidade do Minho, 4710-057, Braga, Portugal
* Corresponding: mfb@isep.ipp.pt
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Abstract: Dinoflagellates are aquatic microorganisms that inhabit both salt and fresh waters. These microorganisms are mostly harmless, however, under certain conditions, some species rapidly reproduce forming water blooms that not only discolor the waters but also compromise the health of every organism in the vicinity, as some dinoflagellates produce potent toxins deemed unsafe for human health (e.g. *Alexandrium minutum*). In this work, a disposable electrochemical genosensor for the detection of the toxic dinoflagellate *Alexandrium minutum* was developed. The analytical platform methodology consisted in a sandwich format heterogeneous hybridization of complementary DNA sequences assay. The 70 bp *A. minutum*-specific targeting probe, the 45 bp fluorescein isothiocyanate-labelled signaling DNA probe and the 25 bp thiolated-DNA-capture probe were designed, after analyzing public databases. To maximize the complementary DNA hybridization and to avoid the formation of strong secondary structures, a mixed mercaptohexanol (MCH) and self-assembled monolayer (SAM) *A. minutum*-specific DNA-capture probe was immobilized onto disposable screen-printed gold electrodes (SPGE). Using chronoamperometric measurements, the enzymatic amplification of the electrochemical signal was obtained with a concentration range from 0.12 to 1.0 nM, a LD of 24.78 pM with a RSD < 5.2 %. This electrochemical genosensor was successfully applied to the selective analysis of the targeted *A. minutum* specific region of denatured genomic DNA, extracted from toxic dinoflagellates present in the Atlantic Ocean.

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1. Introduction

Rivers, lakes, estuaries, beaches, among others, are major economic and ecological sources for humans and their daily activities (e.g. fisheries, agriculture, tourism, aquaculture, ...) [1]. Nevertheless, the increase of these actions has slowly compromised the fundamental ecological structure of these aquatic ecosystems, turning them into favorable environments for phytoplankton microalgae growth and proliferation. This phenomenon is known as algae blooms [2,3].

Dinoflagellate blooms are a natural and, in most cases, beneficial event, since they increase the available resources to feed fishes and other aquatic organisms, contribute to the reduction of atmospheric carbon emissions and regulate the aquatic nutrient cycle [4].

However, the elevated frequency of these blooms now threatens the normal balance of the aquatic ecosystems due to the increase of water toxicity, seeing that some dinoflagellates produce toxins considered unsafe for human health [1,2]. Among them, the *Alexandrium* genus include the most toxic species. The uncontrolled proliferation of toxic dinoflagellates is designated harmful algal blooms (HAB) [5].

Conventional methods like conventional molecular techniques (Fluorescence in situ hybridization (FISH), Enzyme-linked immunosorbent assay (ELISA) and Polymerase chain reaction (PCR)) have long been applied to monitor the water bioactivity [1,4]. Although effective, these techniques still present some limitations [1,4,6]. Therefore, alternative methods, which are potentially faster and more accurate for the detection of HABs, are necessary.

Taking into consideration an electrochemical sensors' aptitude for miniaturization, fast processing, portability, specifically low operation costs, semi-automated system, as well as the ability to perform simultaneous and multi-analyte analyses that enable the detection of low concentrations with high sensitivity and accuracy, electrochemical genosensors seem to be a promising alternative [7].

To our knowledge, this study reports the first disposable electrochemical genosensor for the sensitive and selective determination of the toxic microalgae *Alexandrium minutum* by targeting a specific 70-mer fragment of the toxic algae's coding sequence. The methodology implies the immobilization of a DNA-capture probe (25-mer) targeting the *A. minutum* gene onto disposable screen-printed gold electrodes (SPGE).

To improve the sensor's selectivity and prevent the formation of strong secondary structures that could hinder the hybridization efficiency, a sandwich hybridization format of the *A. minutum* gene was 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/H_2O_2 system. Enzymatic labelling with monovalent ligands provides an improvement regarding the limits of detection, while, simultaneously, introducing selectivity to the measurement [8].

This genosensor presented the capacity to detection of toxic microalgae by targeting the presence of *A. minutum* in cultures established from live cells isolated from the Atlantic Ocean. Futhermore, this genosensor was also compared to real samples of a non-toxic dinoflagellate (*Lingulodinium polyedrum*, isolated from a red tide that occurred in the Mediterranean Sea in 2019) and human oral epithelium cells. The value of this genosensor as an analytical device to detect the presence of toxic microalgae in aquatic environments, namely fisheries and aquaculture tanks, was demonstrated.

2. Material and methods

2.1. Apparatus and electrodes

Screen-printed gold electrodes (SPGE) (C223BT, DropSpen) from Metrohm were used as the electrochemical transductor. These SPGE are constituted of a gold working electrode (Ø 1.6 mm), a silver pseudo-reference electrode and an auxiliary gold electrode on a flat ceramic chip.

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

An orbital shaker (Automate orbital shaker, Heidolph Roramax), used to avoid the cell sedimentation, a GTC96S thermocycler (Cleavere Scientific Ltd., UK), utilized to amplify the DNA strains extracted by PCR, an ultraviolet (UV) fluorescent transilluminator (GenoSmart, VWR), to observe the electrophorese results, a NanoDrop spectrophotometer NanoDrop (NanoDrop Lit, ThermoScientific), to quantify the extracted genomic DNA, a laminar flux chamber (Telstar, PV-30/70), to prepare the culture mediums and a VMR vortex (VV3 model), a Gryozen centrifuge (model 1248R) and a VWR microcentrifuge (model MicroStar12) were also employed.

2.2. Reagents and solutions

All reagents used during the experiments were of analytical grade, therefore no further purifications were required.

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 all obtained from Sigma-Aldrich. Phosphate-buffered saline (PBS) and anti-fluorescein-peroxidase (anti-FITC-POD) fragments were purchased from ThermoFisher and Roche, respectively, while absolute ethanol, isopropanol and ethanol 96% was acquired from PanReac|AppliChem.

Tissue and cell lysis solution (TCL), MPC protein precipitation reagent (MPC) and Proteinase K from Epicnter, the 1x Green Go Taq® Flexi Buffer (GgTBuffer), Magnesium chloride solution ($MgCl_2$) and Go Taq® G2 Flexi DNA Polymerase (Taq.) were acquired from Promega's PCR kit and the PCR Nucleotide Mix (dNTP) was bought from Fermentas (Germany).

The 20x SSPE was diluted with Milli-Q ultrapure water (18.2 MΩ.cm) from a Millipore purification system (20 mL of 20x SSPE buffer in 180 mL of water) to prepare the 2x SSPE buffer used for this study.

2.3. Oligonucleotide sequences and real samples

The construction and development of the genosensor significantly depends on specificity of the selected probes, therefore a DNA sequence capable of detecting the dinoflagellate *A. minutum* was chosen. Each oligonucleotide sequence (**Table 1**) utilized in this study was purchased from Eurogentec (France) as a lyophilized salt.

The oligonucleotide stock solutions (100 nM) were prepared with Milli-Q ultrapure water and stored at – 20 ºC. The working oligonucleotide solutions were prepared daily by diluting the desired amount of the stock solution in the 2x SSPE buffer.

Alexandrium minutum colonial cells isolated from the Atlantic Ocean and purchased from the Roscoff Culture Collection (RCC3029) (France) were cultivated in 40 mL cell/tissue flasks, under sterile conditions, with artificially salted autoclaved water enriched with a f/2 or a Z8 medium. Both mediums were prepared in the 5 L glass flat bottom glass balloons in the laminar flux chamber. All cultures were maintained under a fluorescent lamp on a 14h:10h light:dark cycle, at 18 ºC ± 1 ºC.

2.4. Electrochemical genosensor design

The developed electrochemical genosensor's design involves, essentially, three steps: (i) a sensing phase; (ii) the sandwich format hybridization; and (iv) the electrochemical detection.

To ensure the oligonucleotide's orientation, a SAM (self-assembled monolayer) interface composed by the linear capture probes and the MCH spacer was arranged. As such, 3 μL of the DNA-capture probe (1 μM) was immobilized onto the working electrode overnight. Then, the modified SPGE were rinsed with SSPE 2x buffer and 3 μL of MCH (1 mM) was added to the electrodes – Sensing phase.

The sandwich format hybridization was obtained in a two-part process. The homogeneous hybridization was the first to occur. This process consisted of the binding of the DNA-signaling probe (0.25 μM) to the DNA target probes suspended in the buffer solution, for 30 min. The resulting solution was then added to the modified working electrode, which leads to the attachment of the target/signaling probe solution to the immobilized DNA-capture probes. After an hour, the SPGE were rinsed once more, with the SSPE 2x buffer.

To measure the amplified electrochemical signal, 1.5 U/mL of POD enzymes in a PBS buffer solution were added onto the modified working electrode surface. Lastly, the electrochemical genosensor was connected to the potentiostat and 40 μL of the TMB/H_2O_2 substrate was applied to the electrode's surface for 1 min. The enzymatic oxidization was detected by chronoamperometry at – 0.1 V, for 60 s. Three replicates were carried out for all measurements.

Table 1 - Oligonucleotide sequences.

Oligonucleotide	Sequence 5′ → 3′	Bp
DNA-Capture	**SHC$_6$OH**–TCTATTGGCTCACGGAATTCTGCAA	25
DNA-Signaling	**FC**–GCACACCTTCAAGCATATCCCGAAGGTGCAAATTACGTTCAAACA	45
Target	TTGCAGAATTCCGTGAGCCAATAGATGTTTGAACGTAATTTGCAC-CTTCGGGATATGCTTGAAGGTGTGC	70

SHC$_6$OH – thiol group; **FC** – fluorescein.

3. Results and discussion

3.1. Selection of DNA probes for sandwich format assay

To construct of the genus-specific genosensor, an oligonucleotide sequence specific to *A. minutum* is necessary. So, for this study, a 70-mer synthetic DNA target fragment able to recognize the *A. minutum* dinoflagellate was selected. This DNA sequence has a secondary structure with a Gibbs energy (ΔG) of – 8.94 kcal/mol, under the assay conditions (T = 25 ºC, [Na+] = 0.298 M) determined online (www.ncbi.nim.nih.gov/blast), suitable for this study [9].

The selected 70-mer synthetic DNA target sequence's complementary sequence was cut in two smaller DNA fragments: a 25 bp DNA-capture probe and a 45 bp DNA-signaling probe (**Table 1**). These probes were also designed to minimize the formation of strong secondary structures, because on a planar surface, like on the SPGE surface, strong secondary structures may hinder the hybridization reaction. Under the same conditions, the best DNA-capture and DNA-signaling structures have a ΔG of – 0.71 kcal/mol and – 7.51 kcal/mol, respectively.

3.2. Optimization of the experimental variables

In this study, the experimental parameters, specifically the concentration of the DNA-capture, DNA-signaling and 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 were all optimized.

The first optimized parameter was the concentration of the DNA-capture probe. Four DNA-capture concentrations ranging from 0.25 to 10 μM were immobilized on the working electrodes surface.

The higher the DNA-capture concentration the higher the measured electrochemical current intensity. Nevertheless, when 10 μM was applied, large background currents were registered. This suggests that high concentrations of DNA-capture form non-specific bindings to the working electrode's surface. As a result, the electrodes with the highest S/B ratio value (S/B = 205) are those immobilized with 1.00 μM of the capture DNA. Henceforth, all optimizations will be using 1.00 μM of the DNA-capture probe.

The optimal concentration and incubation time of the SAM assembled onto SPGE was determined by analyzing the intensity of the electrochemical currents attained after hybridization reaction. Under the same analytical conditions (1.0 μM of the DNA- capture probe and 1.0 nM of DNA target, at 25 ºC), different concentrations of MCH (0.0 to 1.00 mM) incubated for 7.5 to 60 min. were tested. The best S/B ratio value (S/B = 481) was obtained with 0.00 μM of MCH during 15 min. However, considering the repeatability of the results, the second-best S/B ratio value (S/B = 464) obtained with 1.00 mM of MCH during 7.5 min was used for future studies.

For this study, a sandwich hybridization format was adopted. This strategy was utilized because the two independent hybridization events (the homogeneous and heterogenous hybridization reactions) increase the overall selectivity of the assay [8]. The homogeneous hybridization occurs between DNA target and DNA-signaling probes and the heterogeneous hybridization between the DNA target/signaling hybrid (obtained from the homogeneous hybridization) and the capture probes immobilized on the working electrodes' surface.

The incubation time for both the homogeneous and heterogenous hybridization reactions were the following optimized variable. The homogeneous hybridization exabits a

higher cathodic electrochemical current (I_{nc}) as well as the higher S/B ratio (S/B = 396) after a 30 min incubation period. On the other hand, the I_{nc} for the heterogeneous hybridization reaction were all relatively similar, however, the best S/B ratio was obtained after 120 min of incubation (S/B = 320). Nevertheless, this ratio value only corresponds to a 2% increase compared to the 60 min ratio so, to decrease the duration of the whole assays, 60 min was the selected time for future trials.

The cleavage of the secondary structures and, consequential, spontaneous align of two complementary probes is normally facilitated at higher temperatures [8]. In that manner, five temperatures (from 25 °C to 98 °C) were measured during the homogeneous hybridization phase, to verify if increasing the temperature would improve the I_{nc}. Under the optimized conditions for the homogeneous event the best S/B ratio (S/B = 211) was observed when the homogenous hybridization reaction occurred at room temperature (25 °C).

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 I_{nc}) was obtained when 0.25 μM of DNA-signaling probes was utilized. The next optimization was performed with 0.25 μM of DNA-signaling probe.

To determine the influence of the anti-FITC-POD enzyme, various concentrations of antibody (0.25 to 10 U/mL) were incubated on the genosensor, from 15 to 60 min. The highest S/B ratios (S/B = 106) was recorded with the anti-FITC-POD concentration of 1.5 U/mL Meanwhile, the best S/B value (S/B = 193) for the anti-FITC-POD incubation time was obtained when the affinity interaction between the antibody and the duplex was held for 30 min.

3.3. Analytical characteristics

According to the selected experimental conditions, the genosensor's analytical performance was evaluated by chronoamperometry using increasing concentrations (0.06 to 5.00 nM) of the 70 bp synthetic DNA target probe. A linear relationship ($r^2 = 0.9995$) between the blank-subtracted intensity current (I_{net}) and the synthetic target concentration was secured in the 0.12 to 1.00 nM range, with a slope of 2.27 ± 0.03 (μA/nM) and an intercept value 0.16 ± 0.01 (μA).

The detection and quantitation limits (LD and LQ) calculated as three time and ten time the estimated standard deviation from the blank assays divided by the slope value were, respectably, 24.78 and 82.60 pM.

The repeatability, reproducibility, and total precision of the electrochemical genosensor were assessed using five electrode measurements. This SPGE were immobilized with a DNA target concentration of 1.00 nM over a five-day period. The repeatability, reproducibility and total precision values were 5.39%; 4.12% and 5.16%, respectably.

The electrochemical genosensor stability was studied by calculating the variation in the chronoamperometric current during three weeks on storage at 4 °C. The genosensor was found stable with a 8 % loss in the current of the immobilized probe.

3.4. Application of the electrochemical genosensor to real samples

Optimized the analytical parameters, the developed electrochemical genosensor was used to detect the amplified PCR genomic DNA collected from the *A. minutum* colonial cells obtained from RCC. The genomic DNA was extracted according to the "MasterPureTM DNA Purification kit" protocol from Epicenter (2012) and its quality and quantity determined by the NanoDrop spectrophotometer.

The extracted DNA enabled a yield of approximately 50 ng/mL and a purity of 1.8 (calculated from the 260/280 ratio), which is a suitable value for conventional PCR amplifications. The *L. polyedrum* and oral epithelium human cells (noncomplementary DNA to the synthetic target sequence) were also submitted to the exact extraction and amplification process and presented a purity value close to 1.8.

To detect the electrochemical signal produced from the genomic DNA hybridization with the synthetic capture and signaling probes, the amplified fragments had suffered a denaturation process carried out by heating these oligonucleotide sequences to 98 ± 1ºC

for 5 minutes and then cooling them down in an ice bath for another 5 minutes. Then 5 µL of the denatured specific PCR products were mixed with the signaling probes to trigger the homogeneous hybridization reaction, at room temperature (25 °C), for 30 minutes.

Afterwards, the analytical signals were recorded following the same steps used for the genosensors construction. The electrochemical genosensor developed for the detection of the *A. minutum* gene sequence was applied to the analysis of 1 nM of the amplified genomic DNA extracts from the *A. minutum* artificially grown colonial, *L. polyedrum* and human (oral epithelium) cells.

3.5. Selectivity and Sensitivity

The selectivity of the hybridization strategy was guaranteed when comparing the chronoamperometric responses acquired in the absence and in the presence of 1 nM of the synthetic target DNA (DT), two complementary DNA extracted from the *A. minutum* colonial cells (A1 and A2, diluted to a concentration of 1nM) and two noncomplementary (NCLP and NCH) DNA sequences. To verify the sensor's selectivity, the complementary and noncomplementary samples were diluted to a concentration of 1 nM (**Fig. 1A**). The highest I_{nc} were attained for the synthetic DNA target sequence (DT) and genomic DNA (A1 and A2) extracted from the *A. minutum* cells. On the other hand, the I_{nc} from the NCLP (the L. polyedrum) and NCH (human) sequences was similar to their blanks. These results suggest that the adopted format is a viable option to identify toxic algae in aquatic environments.

To confirm the sensors sensitivity, the A1 and A2 samples were diluted 5x, 10x, 20x and 50x from the original extraction (**Fig. 1B**). The four dilutions show different electrochemical signals from the blank assay. Thus, even with the highest dilution factor (1:50) the electrochemical signal was 15x higher than the I_{nc} obtained by the blank assays. This signifies that the electrochemical genosensor responds to different concentrations of the amplified colonial *A. minutum* DNA with no visible interferences from the PCR reagents.

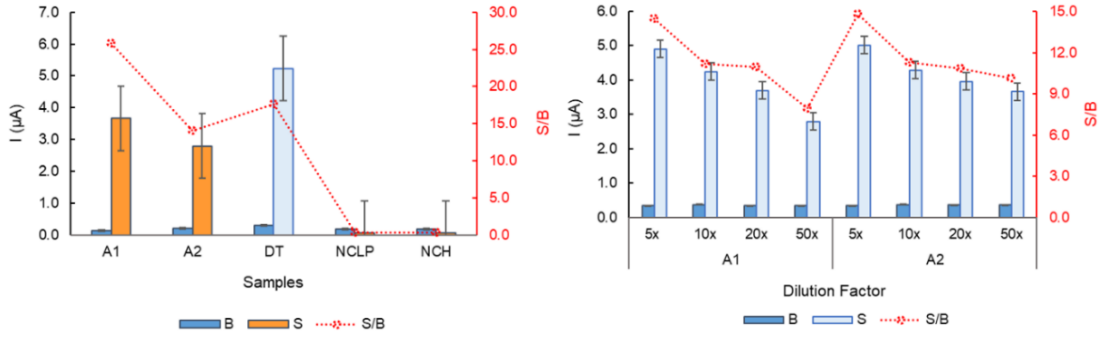

Figure 1 - Comparison of the electrochemical detection signal of **A)** the complementary (A1 and A2) and non-complementary (NCLP and NCH) amplified DNA to the synthetic DNA target (DT) and **B)** the dilution factor of the amplified A1 and A2 *Alexandrium minutum* DNA cultures. Current values of the blank assays (B) represented in dark blue, signal (S) in light blue and orange and the corresponding S/B ratio in red. 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, at 25 ºC; heterogeneous hybridization incubation time = 60 min; concentration and incubation time of the DNA-signaling probe = 0.25 µM, during 30 min; and concentration and incubation time of the anti-FITC-POD antibody = 1.5 U/mL, for 30 min. Error bars estimate the standard deviation of three replicates.

4. Conclusions

The developed disposable electrochemical genosensor presents a high selectivity and sensibility when detecting both the synthetic and the genomic DNA of the toxic *A. minutum* dinoflagellates. It is also capable of detecting these DNA sequences in various concentrations.

All optimized parameters contributed to enhance the sensor's sensitivity, resulting in its reduced detection limit. Therefore, the electrochemical genosensor exhibited a good performance, with an LD and LQ of 24.78 pM and 82.60 pM, respectively, as well as a high repeatability (5.39 %) and reproducibility (4.12 %).

The adopted genosensor design permitted high sensitivity and selectivity was achieved by creating a mixed SAM compound (composed of thiol-DNA-capture probe and MCH) and by using a sandwich format assay, respectably. The amplification of the electrochemical signal conducted by the (POD) enzyme also contributed for the developed genosensor's good performance.

Using the DNA extracted and amplified by PCR, from three different biological samples (pure cultures of *A. minutum*, *Lingulodinium polyedrum* dinoflagellates, from the red tide that occurred in Algarve, Portugal, on June 16 th 2019, and human oral epithelium cells) allowed to validate the designed genosensor's selectivity and sensitivity because it detected the complementary DNA from the *A. minutum* cultures (complementary DNA to oligonucleotides used in sensor construction), while not perceiving the DNA from the noncomplementary samples (i.e., *L. polyedrum* and oral epithelium cells). On the other hand, the detection of different DNA dilutions of the *A. minutum* cultures confirmed its sensitivity.

Therefore, the simplicity in handling these sensors, as well as their high sensitivity and selectivity, makes them a promising analytical tool to monitor algae blooms, namely *A. minutum* blooms, in distinct aquatic ecosystems, including fisheries and aquaculture tanks.

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