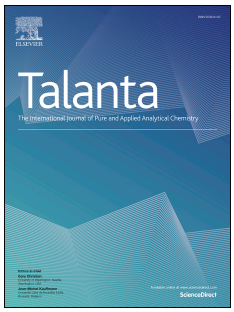

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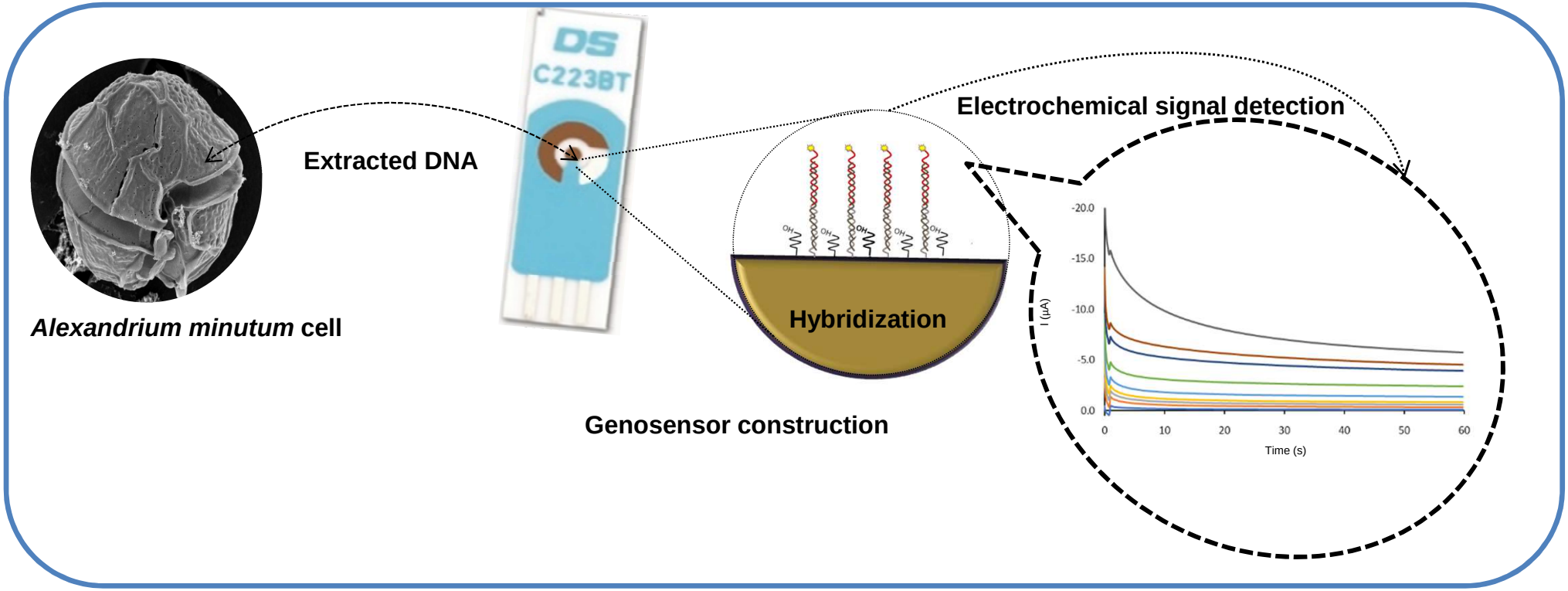

Alexandrium minutum **cell**

Electrochemical genosensor for the detection of *Alexandrium minutum* dinoflagellates

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Abstract

This work addresses the development of a disposable electrochemical genosensor for the detection of the toxic dinoflagellate, *Alexandrium minutum*. Analyzing public databases, a specific 70 bp DNA probe, targeting *A. minutum*, was selected and designed. The genosensor methodology implied the immobilization of a *A. minutum*-specific DNA-capture probe onto screen-printed gold electrodes (SPGE). To improve both the selectivity and to avoid strong secondary structures, that could hinder the hybridization efficiency, a sandwich format of the *A. minutum* gene was designed using a fluorescein isothiocyanate-labelled signaling DNA probe and enzymatic amplification of the electrochemical signal.

Using this electrochemical genosensor, a concentration range from 0.12 to 1.0 nM, a LD of 24.78 pM with a RSD < 5.2 % was determined. The genosensor was successfully applied to the selective analysis of the targeted *A. minutum* specific region denatured genomic DNA extracted from toxic dinoflagellates present in the Atlantic Ocean.

Keywords: *Alexandrium minutum*; Dinoflagellate; Chronoamperometry; Electrochemical genosensor; Sandwich format hybridization.

1. INTRODUCTION

Aquatic environments such as rivers, lakes, estuaries, coastal areas are important economic and ecological sources for human activities (e.g. fisheries, tourism, agriculture and aquaculture) [1]. However, the increase in those practices has, over the years, compromised the integrity of these ecosystems. Runoffs of terrestrial nutrients (from, for example, agricultural and industrial waste) and higher surface temperatures are believed to have transformed these ecosystems into favorable habitats for algae growth and proliferation. As a result, the frequency in phytoplankton microalgae blooms is rising worldwide [2-4].

Phytoplankton microalgae are a heterogeneous group of organisms (namely diatoms, dinoflagellates and cyanobacteria) that compose the base of all aquatic food webs. Their blooms are a relatively natural and, in some cases, beneficial phenomenon, for fisheries and other practices. The latter are also of critical importance to the environment since they contribute for the fixation of half of Earth's carbon and regulate the aquatic nutrients cyclic [5-9].Nevertheless, the continuous increase of bloom events now pose a threat to the normal balance of those aquatic ecosystems due to the reduction of the water's oxygen levels, light conditions or toxicity, given that some species are capable of producing harmful toxins [1,2,10,11]. The uncontrolled proliferation of these potentially hazard species is designated harmful algal blooms (HAB). Among the toxic microalgae, the dinoflagellates from the *Alexandrium* genus include the most toxic species [1,12].

From public safety risks to economic and ecological losses (due to the decrease in tourism, port and recreational activities and the contamination of local wildlife and water supplies), the occurrence of a HAB is always negatively viewed because of the socioeconomic impacts they have over a community. Still, their biggest threat results from the accumulation of toxins in other organisms, such as fishes, birds and shellfish, that can find their way through the food web to humans [1,3]. Ciguatoxins (responsible for ciguatera poisoning), saxitoxins (responsible for paralytic shellfish poisoning (PSP)), brevetoxins (responsible for neurotoxin shellfish poisoning (NSP)), okadaic acid (responsible for diarrheic shellfish poisoning (DSP)) and domoic acid (responsible for amnesic shellfish poisoning (ASP)) are five of the most common HAB related toxins worldwide. In temperate regions, such as, Europe, Asia, Australia, South Africa and North and South America, HAB generally cause ASP, DSP, NSP, PSP and azaspiracid

(AZP) outbreaks [8,13]. Every year, an average of sixty thousand people present HAB toxin intoxication symptoms, with a mortality estimate rate of 1.5% [14,15]. Therefore, the early detection of these organisms has become a major concern.

Bioassays have long been the standard methodology to determine the effect of a chemical or biological event. However, since 2012, Europe has applied viable alternatives, like the conventional molecular techniques (Fluorescence in situ hybridization (FISH), Enzyme-linked immunosorbent assay (ELISA) and Polymerase chain reaction (PCR)) to monitor the water's bioactivity [1,8]. While effective, these techniques still present some limitations. The use of conventional methods (to monitor toxic microalgae) can be extremely time consuming, require expensive equipment and trained personnel. Some also call for sample transportation and specialized laboratories [1,8,16-18]. Thus, other methods, which are potentially faster and more accurate for the detection of HABs, namely the application of electrochemical genosensors, seems to be a promising alternative.

Taking into consideration an electrochemical sensors' aptitude for miniaturization, they present several advantages to the conventional molecular techniques, specifically their low operation costs, fast processing, portability, 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, even in turbid samples [19,20].

Until now, some DNA oligonucleotides electrochemical genosensors have been developed allowing the identification of toxic microalgae, namely *Prymmnesium parvum* [21], *Alexandrium tamarense* [22] and *Gymnodinium catenactum* [23].

This work reports the first disposable electrochemical genosensor for the sensitive and selective determination/identification of the toxic microalgae *Alexandrium minutum* by targeting a specific 70-mer fragment of its 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 selectivity, as well as to avoid strong secondary structures that can 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 (**Scheme 1**). Enzymatic labelling with monovalent ligands provides an improvement regarding the limits of detection, while, simultaneously, introducing selectivity to the measurement [24].

The developed electrochemical genosensor was successfully applied for the detection of toxic microalgae by targeting the presence of *A. minutum* in cultures established from live cells isolated from the Atlantic Ocean. Additionally, the developed genosensor was also compared to a live sample of a non-toxic microalgae found in a HAB and human oral epithelium cells.

The utility of this approach as an analytical device to be used in aquatic environments, namely fisheries and aquaculture to screen for the presence of toxic microalgae, was demonstrated.

2. MATERIAL AND METHODS

2.1. Apparatus and electrodes

Screen-printed gold electrodes (SPGE) (C223BT, DropSpen) purchased from Metrohm were used 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 of L 33 x W 10 x H 0.5 mm. The 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 $\pm$ 0.5 ºC).

The *Alexandrium minutum* cell cultures were grown in 5 L glass bottom flat balloons. A laminar flux chamber (Telstar, PV-30/70) was used when preparing the culture mediums.

An orbital shaker (Automate orbital shaker, Heidolph Roramax) to prevent the cell sedimentation, a GTC96S thermocycler (Cleavere Scientific Ltd., UK) to amplify the DNA strains extracted by PCR, an ultraviolet (UV) fluorescent transilluminator (GenoSmart, VWR) to observe the electrophorese results, a NanoDrop spectrophotometer (NanoDrop Lit, ThermoScientific) to quantify the extracted genomic DNA and a VMR vortex (VV3 model), Gryozen centrifuge (model 1248R) and VWR microcentrifuge (model MicroStar12) were also employed.

2.2. Reagents, solutions and samples

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

6-mercapto-1-hexanol (MCH), bovine serum albumin (BSA), 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) and the anti-fluorescein-peroxidase (anti-FITC-POD) fragments were obtained from ThermoFisher and Roche, respectively.

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 from Promega's PCR kit and the PCR Nucleotide Mix (dNTP) was acquired from Fermentas (Germany).

The 20x SSPE was diluted with Milli-Q ultrapure water (18.2 MΩ.cm) from a Millipore purification system in a 1:9 ratio (20 mL of 20x SSPE buffer in 180 mL of water) to prepare the 2x SSPE buffer used in this study.

Alexandrium minutum colonial cells, isolated from the Atlantic Ocean, were acquired from the Roscoff Culture Collection (RCC3029) (France) and grown under sterile conditions in 40 mL cell/tissue flasks with artificially salted autoclaved water enriched in f/2 and Z8 medium. Both mediums were prepared in the laminar flux chamber in the 5 L flat bottom glass balloons. All cell cultures were maintained under a fluorescent lamp with a 14h:10h light:dark cycle at a constant temperature of 18 ºC $\pm$ 1 ºC. Details of the culture mediums (f/2 and Z8) are described in the supporting information.

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 used in this experiment was bought from Eurogentec (France) as a lyophilized salt (**Table 1**).

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

The capture probe was functionalized with a thiol group at the 5' end to facilitate its attachment onto the gold substrate, while the signaling probe was functionalized with a

fluorescein at the 3' end. These two probes are completely complementary to the target sequence, forming a perfect and rigid duplex.

The capture, signaling and target sequences structures are represented in **Fig. S1** in the supplementary information.

2.3. Electrochemical genosensor design

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

Before use, all SPGE were washed with ethanol and water and dried with a nitrogen flow (Pretreatment).

To ensure the orientation of the probes, a SAM interface consisting of linear capture probes and MCH was arranged. First, 3 µL of the DNA-capture probe (1 nM) was immobilized onto the working electrode and stored in a humified Petri dish overnight. Afterwards, the modified SPGE was rinsed twice with 200 µL of the SSPE 2x buffer, removing the weakly attached probes and 3 µL of MCH (1 µM) was added to the electrodes (Sensing phase).

The sandwich format assay was attained in a two-step hybridization. First, the homogeneous hybridization takes place when the DNA-signaling probe (0.25 µM) binds to the DNA target in the buffer solution, for 30 min. Then, the resulting solution was added to the modified electrode which leads to the binding of the target/signaling probe solution to the immobilized DNA-capture probes. After an hour, the electrodes were rinsed to remove any nonspecific adsorbed sequences.

For the real samples, an intermediate step was required. Before the addition of the target, the amplified DNA was heated at 95 ºC for 5 min, cooled in ice for another 5 min and then left at room temperature (25 °C) in the remaining time (Hybridization).

The sandwich hybridization format increases the selectivity of the assay since two independent hybridization events take place, the homogeneous one between the target and the signaling probe 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 onto the working electrode surface. The electrode was rinsed again 30 min later.

Finally, the genosensor was connected to the potentiostat and 40 µL of the TMB/H_2O_2 substrate was added to cover the electrode for 1 min (**Fig. S2**). The detection of the

enzymatically oxidized product was performed by chronoamperometry at – 0.1 V, for 60 s. Three replicates were carried out for all measurements.

Details about all the protocols used (genomic DNA and *Alexandrium minutum* culture cells' preparation) are described in detail in the supporting information.

3. RESULTS AND DISCUSSION

3.1. Selection of DNA probes for sandwich format assay

For the construction of the genus-specific electrochemical genosensor, a DNA oligonucleotide sequence specific to the dinoflagellate *A. minutum* is required. In this study, a 70-mer synthetic DNA target fragment capable of identifying the *A. minutum* microalgae was selected. This oligonucleotide sequence has 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), which is suitable for this genosensing study [25].

The complementary sequence (to the designed synthetic target probe) was cut, generating two smaller DNA fragments: a 25 bp DNA-capture probe and a 45 bp DNA-signaling probe. The capture and signaling probes were also designed to minimize the formation of secondary structures, since on a planar surface (such as the gold electrode) strong secondary structures may hinder the hybridization process. The best DNA structures present a ΔG of – 0.71 kcal/mol (for the DNA-capture probe) and - 7.51 kcal/mol (for the DNA-signaling probe), under the same conditions. These values indicate that there is not only a smaller probability of forming strong secondary structures (low ΔG) but also have a higher chance of the three probes spontaneously hybridizing. All sequences are shown in **Table 1** and their molecular structures are represented in the supplementary material (**Fig. S1**).

3.2. Selection of the disposable screen-printed gold electrode

The experimental variables involved in the biosensor development were optimized taking as the selection criterion the largest ratio between the chronoamperometric

signals measured at – 0.1 V (vs. the Ag pseudo-reference electrode) for 0 (blank, B) and 1 nM of synthetic target DNA (signal, S) (signal-to-blank, S/B, ratio).

For this study, disposable SPGE were chosen as the electrochemical transductor since gold is (1) a highly conductible metal; (2) biocompatible to form self-assembled monolayers (SAMs); (3) the most common metal used in genosensors' design [26].

Two types of SPGE are available in the market; the SPGE with high (AT) and low (BT) temperature curing inks. Considering that these SPGE may present different properties depending on the application, the effect of using SPGE-AT or SPGE-BT was evaluated by analyzing the efficiency of the hybridization through recording the cathodic current of the TMB enzymatically oxidized.

Fig. 1 displays the electrochemical measurements obtained when the SPGE-AT and SPGE-BT electrodes were immobilized with distinct DNA-capture probe concentrations (1 and 10 nM). Analyzing **Fig. 1**, it is possible to verify that higher chronoamperometric currents were obtained when the SPGE-BT was used (1.90 and 2.64 -fold more). In fact, using this electrode, a S/B ratio of 11.3 and 40.3 were calculated when a concentration of 1 nM and 10 nM of DNA-capture probe was used, respectively. Moreover, when the SPGE-AT was used, large background currents (blank signal) were measured, suggesting a high susceptibility to nonspecific adsorption [27]. Therefore, the SPGE-BT were used for the subsequent studies.

3.3. Optimization of the experimental variables

Most of the experimental parameters, namely 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 involved in the genosensor development were optimized.

To determine the influence of the DNA-capture probe concentration in the intensity of the electrochemical currents, four DNA-capture concentrations ranging from 0.25 to 10 μM were immobilized on the SPGE-BT working electrodes surface.

Fig. 2 reveals the effect of increasing the concentration of the DNA-capture probe immobilized onto the SPGE-BT. As shown, increasing the DNA-capture probe concentration intensifies the electrochemical current measured after the hybridization

reaction. Nevertheless, when 10 μM of DNA-capture probe was used, large background currents were obtained. This result suggests that high concentrations of the DNA-capture probe form nonspecific bindings on the electrodes surface [27]. As a result, the electrodes with the highest S/B ratio value of 205 are those immobilized with 1.00 μM of the capture DNA. Future optimizations will proceed using 1.00 μM of the DNA-capture probe.

SAM of functionalized thiols groups on gold substrates are frequently applied in biochemical sensing because the sulfur–gold bonds are (1) incorporate well with other functional groups; (2) are easy to prepare; and (3) present high stability and reproducibility due to their covalent bond [21,28-30]. According to Campuzano et al., better analytical signals can be obtained if DNA probes are linearly oriented as SAM on a gold platform, since the preferential orientation adopted by the thiol group can minimize the nonspecific adsorption of biomolecules while facilitating the permeability for small molecules, such as TMB [21,27]. Thus, an MCH spacer was applied to form a SAM composed of the thiolated DNA-capture probe and the MCH spacer on the electrodes' surface.

In this work, the concentration and incubation time of the SAM assembled onto SPGE-BT were optimized by analyzing the intensity of the electrochemical currents obtained 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 ranging from 0.0 to 1.00 μM incubated over short periods of time, 7.5 to 60 min., were tested (**Fig. 3A to 3D**). The best S/B ratio value was obtained when the SPGE-BT were incubated with 0.00 μM of MCH during 15 min (S/B = 481) (**Fig. 3B**). This result suggests that it is possible to continue this study without the addition of the MCH spacer. In this case, it is believed that the DNA-capture probes have organized themselves in SAMs since they only possess a 25-mer sequence. However, considering the repeatability of the results, the second-best S/B ratio value (S/B = 464) obtained when MCH concentration = 1.00 μM and incubation time = 7.5 min (**Fig. 3A**) was used for future studies.

In this work, a sandwich hybridization format was adopted. This strategy was used because the two independent hybridization events (the homogeneous and heterogenous hybridization) increase the overall selectivity of the assay [24]. In this case, the homogeneous hybridization results from the partial hybridization between the DNA target and DNA-signaling probes and the heterogeneous hybridization between

the target-signaling hybrid and the capture probes fixated on the electrodes surface (**Scheme 2)**.

The incubation time for both hybridization steps were optimized (**Fig. 4A** and **4B**). For the homogeneous hybridization, the highest cathodic electrochemical current (Inc) as well as the best S/B ratio (S/B = 396), was obtained after 30 min of incubation (**Fig. 4A**). As for the heterogeneous hybridization reaction, each tested interval presented a similar Inc, however, as observed in **Fig. 4B**, as the incubation period increases so does the S/B ratio, hence the best ratio was obtained after 120 min of incubation (S/B = 320). Nevertheless, this ratio value only corresponds to a 2% increase when compared to the 60 min ratio so, in order to decrease the duration of the whole assays, 60 min was the selected time for the future trials.

For the homogeneous hybridization reaction to take place, both the DNA target and the DNA-signaling probes must break their secondary structures (**Fig. S1**) and align spontaneously to the other's complementary bases. This process is normally facilitated if those probes are succumbed to higher temperatures [21]. In that manner, five temperatures (from 25 °C to 98 °C) were applied to the reaction, during the homogeneous hybridization phase, to verify if increasing the temperature would improve the genosensor performance (**Fig. 4C**). Under the optimized conditions for the homogeneous event (1.0 μM of DNA-capture probe, 1.0 nM of DNA target and homogenous hybridization incubation time of 30 min) the best S/B ratio (S/B = 211) was observed when the homogenous hybridization reaction occurred at 25 °C. On the other hand, the addition of BSA – a protein frequently used as a blocking agent to avoid unspecific banding onto the electrode surface – did not enhance the genosensor's performance (**Fig. 4D**), i.e., at room temperature (25 °C) and under the previous conditions for the homogeneous hybridization step, the best S/B ratio was obtained in the absence of BSA (S/B = 398). These results are believed to be influenced by the selected DNA probes; the higher the ΔG of an oligonucleotide sequence, the more likelihood of it forming secondary structures, however, since the designed synthetic probes (DNA-capture, -signaling and target) have relatively low ΔG, it's unlikely that strong secondary structures may occur. In this case, the increasing temperatures and BSA protein will only interfere with the hybridization process instead of optimizing its efficiency.

Therefore, the next experimental parameters optimizations were performed at room temperature (25 °C) and in the absence of BSA.

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. As can been seen in **Fig. 5**, the best S/B ratio (as well as the highest Inc) 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.

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 amount of hybridized sequences on the electrode's surface [24]. In order to determine its influence on the genosensors performance, several concentrations of antibody ranging from 0.25 to 10 U/mL were incubated on the genosensor (**Fig. 6A**), over an extended period of time: 15 to 60 min (**Fig. 6B**). Considering that the electrochemical current of the blank assay did not change significantly, higher S/B ratios were obtained when increased concentrations of the anti-FITC-POD enzyme were added to the genosensor. S/B ratios values of 16.6, 64.1, 96.6, 106 and 104 were recorded for 0.25; 0.5; 1.0; 1.5 and 10 U/mL, respectively. Thus, 1.5 U/mL of anti-FITC-POD was the concentration selected.

In regard to the anti-FITC-POD incubation time, the best S/B ratio (193) was obtained when the affinity interaction between the antibody and the DNA duplex was held for 30 min.

Table 2 summarizes all the selected experimental parameters as well as the test ranges in which they were optimized.

3.4. Analytical characteristics

Under the selected experimental conditions (**Table 2**) the developed genosensor's analytical performance was assessed by chronoamperometry using increasing concentrations (0.06 to 5.00 nM) of the 70-mer synthetic DNA target probe. The resulting calibration plot can be found in the supporting information (**Fig. S2**). A linear relationship (r^2 = 0.9995) between the blank-subtracted intensity current (I_{net}) and the synthetic target concentration was obtained in the 0.12 to 1.00 nM range, with a slope and intercept value of 2.27 $\pm$ 0.03 (µA/nM) and 0.16 $\pm$ 0.01 (µA), respectively.

The detection and quantitation limits (LD and LQ), calculated as three time and ten time the estimated standard deviation from the blank assays (chronoamperometric measurements registered in the absence of target DNA) divided by the value of the linear calibration plot slope were, respectably, 24.78 and 82.60 pM.

The repeatability, reproducibility and total precision of the electrochemical genosensor were determined using five electrodes measurements immobilized with a DNA target concentration of 1.00 nM over a five-day period. The repeatability, reproducibility and total precision values were, respectively, 5.39%; 4.12% and 5.16%, as shown in **Table 3**.

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

3.5. Application of the electrochemical genosensor to the analysis of natural samples

After the selection of the optimized analytical parameters, the proposed 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 (260/280 ratio) of 1.8, which is an adequate value for conventional PCR amplifications. *L. polyedrum* and oral epithelium human cells (which act as control and noncomplementary DNA) were also submitted to the exact extraction and amplification process. The genomic DNA extracted from those cells also presented a purity value close to 1.8.

An 2% agarose gel electrophorese was employed to determine the length of the amplified DNA fragments. It´s results were obtained by the fluorescent UV transilluminator (**Fig. S3**).

Additional details about the cell’s extraction, amplification and quantification can be found in the supplementary information.

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 ± 1 ºC for 5 minutes and then cooling them down in an ice bath for another 5 minutes. This is a compulsory step due to the DNA's natural double stranded nature [24]. 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 *Alexandrium 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.

3.5.1. Selectivity and Sensitivity

The selectivity of the adopted hybridization strategy was verified by comparing the chronoamperometric responses obtained 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 47x and 48x respectively, in order to obtain a DNA concentration of 1nM) and two noncomplementary (NCLP and NCH) DNA sequences. To corroborate the sensor's selectivity, the complementary and noncomplementary samples were also diluted to a concentration of 1 nM. **Fig. 7** reveals the results acquired in this study.

As it is seen, the highest Inc (3.0 to 5.0 µA) were obtained for the synthetic DNA target (DT) and DNA extracted sequences (A1 and A2) of *A. minutum*. On the other hand, NCLP (the *L. polyedrum*) and NCH (human) sequences produced an electrochemical signal in the same range as their blanks. These results suggest that the adopted hybridization scheme is a viable option to discriminate (at least) between the genus of toxic algae.

To confirm the sensors sensitivity, the A1 and A2 samples were diluted 5x, 10x, 20x and 50x from the original extraction.

As seen in **Fig. 8** the four dilutions presented distinguished electrochemical signals from the blank assay. Thus, even with the highest dilution factor (1:50) the electrochemical signal was 15 times higher than the Inc obtained by the blank assays.

These results indicate that the electrochemical genosensor responds to different concentrations of amplified DNA without the interference from the PCR reagents.

4. CONCLUSIONS

The developed disposable electrochemical genosensor is capable of detecting, with high selectivity and sensibility, the synthetic and genomic DNA (in various concentrations) of the toxic microalgae *A. minutum*.

The construction of this genosensor was attained by creating a mixed SAM compound (composed of thiol capture DNA and MCH) on the working electrodes' gold surface. In order to increase selectivity, the hybridization reaction was performed in sandwich format, the amplification of the electrochemical signal conducted by an enzyme (POD) and chronoamperometry was used to measure the electrical currents.

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

Using the DNA extracted and amplified by PCR, from 3 distinct biological samples (pure cultures of *A. minutum*, *Lingulodinium polyedrum* dinoflagellates, from the red tide that occurred in Algarve, Portugal, on June 16th 2019, and human oral epithelium cells) allowed to validate and enhance the selectivity and sensitivity of the genosensor.

The genosensor also proved to have a high selectivity, as 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 did not developed any analytical response; electrochemical signals similar to the blank assays). On the other hand, the detection of different DNA dilutions of the *A. minutum* cultures confirmed its sensitivity.

Thus, the ease and simplicity in handling these sensors, as well as their high sensitivity and selectivity, makes them a promising portable, user-friendly and low-cost analytical tool to monitor the presence of *A. minutum* dinoflagellates in all sorts of aquatic environments, including aquaculture tanks.

Supporting Information available: The following files are available free of charge. Supp Info. Figure S1. Possible folding of the oligonucleotide sequences calculated in unafold.rna.albany.edu (temperature = 25ºC; [Na+] = 0.298 M; [Mg] = 0.000 M), Figure S2 – Cyclic voltammograms of TMB in a) bare SPGE and b) modified electrode; Table S1 – Recipe of the f/2 culture medium (adapted from Guillard & Ryther, 1962; Guillard, 1975), Table S2 – Recipe for the Z8 culture medium, Figure S3 – Agarose gel (2%) electrophoresis detection of the amplified genomic DNA samples. L = ladder, A1 and A2 = *Alexandrium minutum* colonial samples; Alg = *Lingulodinium polyedrum* colonial cells; and B = blank.

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TABLE CAPTIONS:

Table 1 – Oligonucleotide sequences.

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Table 3 – Analytical parameters of the developed electrochemical genosensors.

FIGURE CAPTIONS:

Scheme 1 – General procedure for the development of an electrochemical genosensor.

Scheme 2 - General scheme of the sandwich format assay. DNA-Capture, DNA target, DNA-signaling and MCH probes represented in light blue, grey, orange and black, respectably. The fluorescein protein is exemplified in yellow.

Figure 1 – Chronoamperometric responses obtained when studying the influence of the type of electrode (SPGE-AT or SPGE-BT) with two different concentrations of the DNA-capture probe (1 and 10 μM). Blank (B) values represented in dark blue, signal (S) in light blue and the corresponding S/B ratio in red. Analytical parameters: DNA target concentration = 1.0 nM; concentration and incubation time of MCH = 1.0 μM, during 30 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

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Figure 4 – Influence of the incubation time in the (A) homogeneous and (B) heterogenous hybridization and the effect of the (C) homogenous hybridization temperature and (D) addition of BSA in the homogenous hybridization phase. Blank (B) values represented in dark blue, signal (S) in light blue and the corresponding S/B ratio in red. Analytical parameters: concentration of the DNA-capture probe = 1.0 µM; DNA target concentration = 1.0 nM; concentration and incubation time of MCH = 1.0 µM, for 7.5 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 = 1.0 U/mL, for 30 min. Error bars estimate the standard deviation of three replicates

Figure 5 – Chronoamperometric responses acquired when optimizing the concentration of the DNA-signaling probe. Blank (B) values represented in dark blue, signal (S) in light blue and the corresponding S/B ratio in red. Analytical parameters: concentration of the DNA-capture probe = 1.0 μM; DNA target concentration = 1.0 nM; concentration and incubation time of MCH = 1.0 μM, for 7.5 min; homogeneous hybridization incubation time = 30 min, at 25 ºC; heterogeneous hybridization incubation time = 60 min; and concentration and incubation time of the anti-FITC-POD antibody = 1.0 U/mL, for 30 min. Error bars estimate the standard deviation of three replicates.

Figure 6 – Chronoamperometric responses acquired when optimizing the concentration (A) and the incubation time (B) of the antibody Anti-FITC-POD. Blank (B) values represented in dark blue, signal (S) in light blue and the corresponding S/B ratio in red. Analytical parameters: concentration of the DNA-capture probe = 1.0 μM; DNA target concentration = 1.0 nM; concentration and incubation time of MCH = 1.0 μM, during 7.5 min; homogeneous hybridization incubation time = 30 min, at 25 ºC; heterogeneous hybridization incubation time = 60 min; and concentration and incubation time of the DNA-signaling probe = 0.25 μM, during 30 min. Error bars estimate the standard deviation of three replicates.

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Table 1 – Oligonucleotide sequences.

OLIGONUCLEOTIDE	SEQUENCE 5´→ 3´	BP
DNA Capture probe	**SHC_6OH–**TCTATTGGCTCACGGAATTCTGCAA	25
DNA Signaling	**FC–** GCACACCTTCAAGCATATCCCGAAGGTGCAAATTACGTT CAAACA	45
DNA Target	TTGCAGAATTCCGTGAGCCAATAGATGTTTGAACGTAAT TTGCACCTTCGGGATATGCTTGAAGGTGTGC	70
Primer 1	TTGCAGAATTCCGTGAGCCA	20
Primer 2	GCACACCTTCAAGCATATCCC	21

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

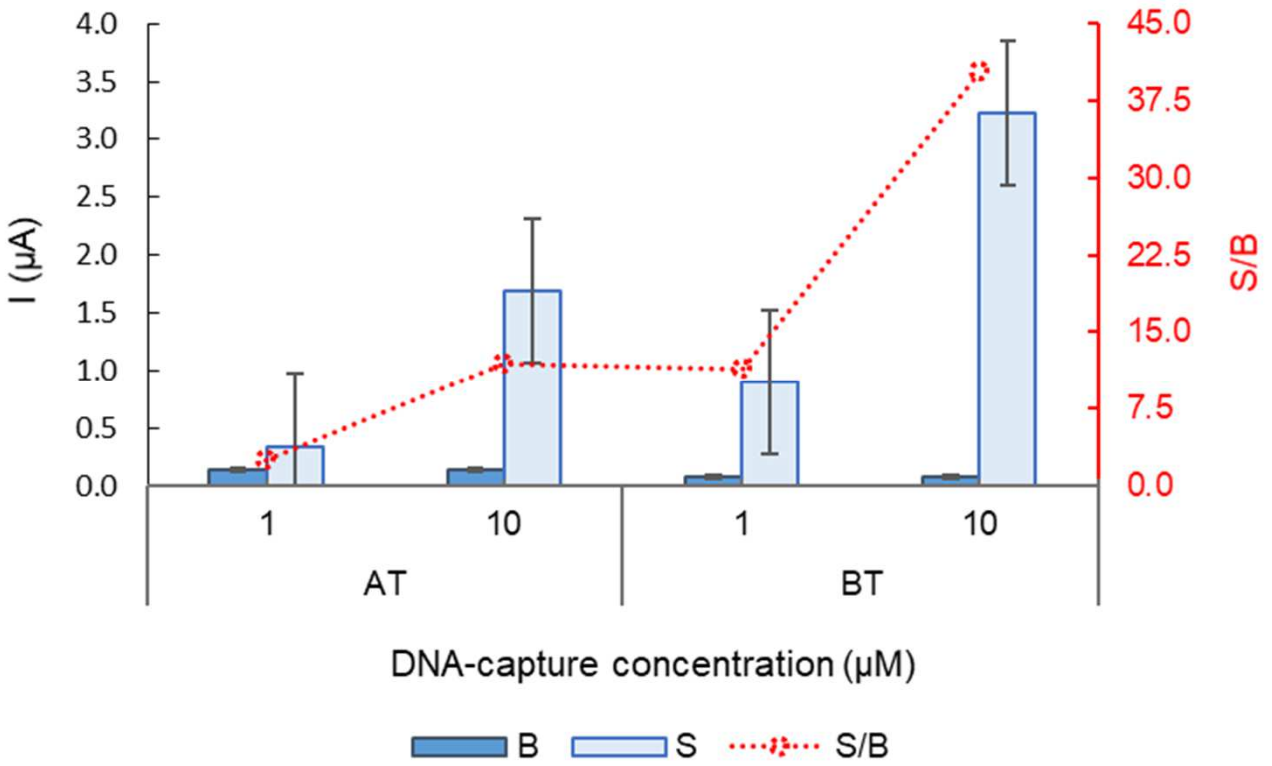
Table 2 – Selected values for the electrochemical genosensor construction.

Variables	Tested range	Selected value
DNA Capture probe concentration (μM)	0.25 – 10.0	1.0
MCH concentration (μM)	0.0 – 1.0	1.0
MCH incubation time (min)	0 – 60	7.5
Homogeneous hybridization incubation time (min)	15 – 60	30
DNA-signaling concentration probe (μM)	0.13 – 0.50	0.25
Antibody concentration (U/mL)	0.25 – 10.0	1.5
Antibody incubation time (min)	15 – 60	30
Heterogeneous hybridization incubation time (min)	30 – 120	60
Homogenous hybridization temperature (°C)	25 ; 98	25
BSA (presence +; absence -)	+ BSA ; - BSA	- BSA

Table 3 – Analytical parameters of the developed electrochemical genosensors.

Parameters	**Results**
Linearity (nM)	0.12 – 1.00
Slope	2.27
Interception	0.16
Correlation (R)	0.999
Slopes' standard deviation	0.03
Interceptions' standard deviation	0.01
LD (pM)	24.78
LQ (pM)	82.60
Repeatability [1] (%)	5.39
Reproducibility [1] (%)	4.12
Total precision (%)	5.16

[1] n = 5



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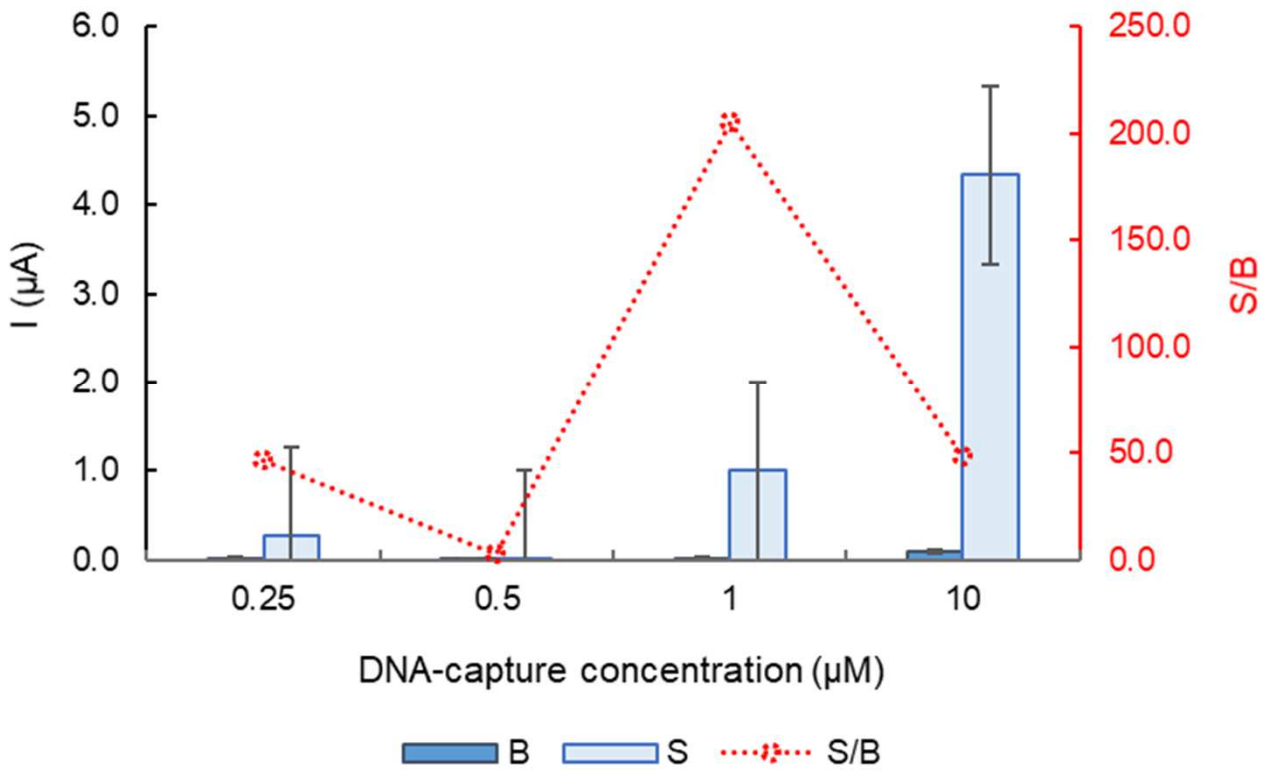

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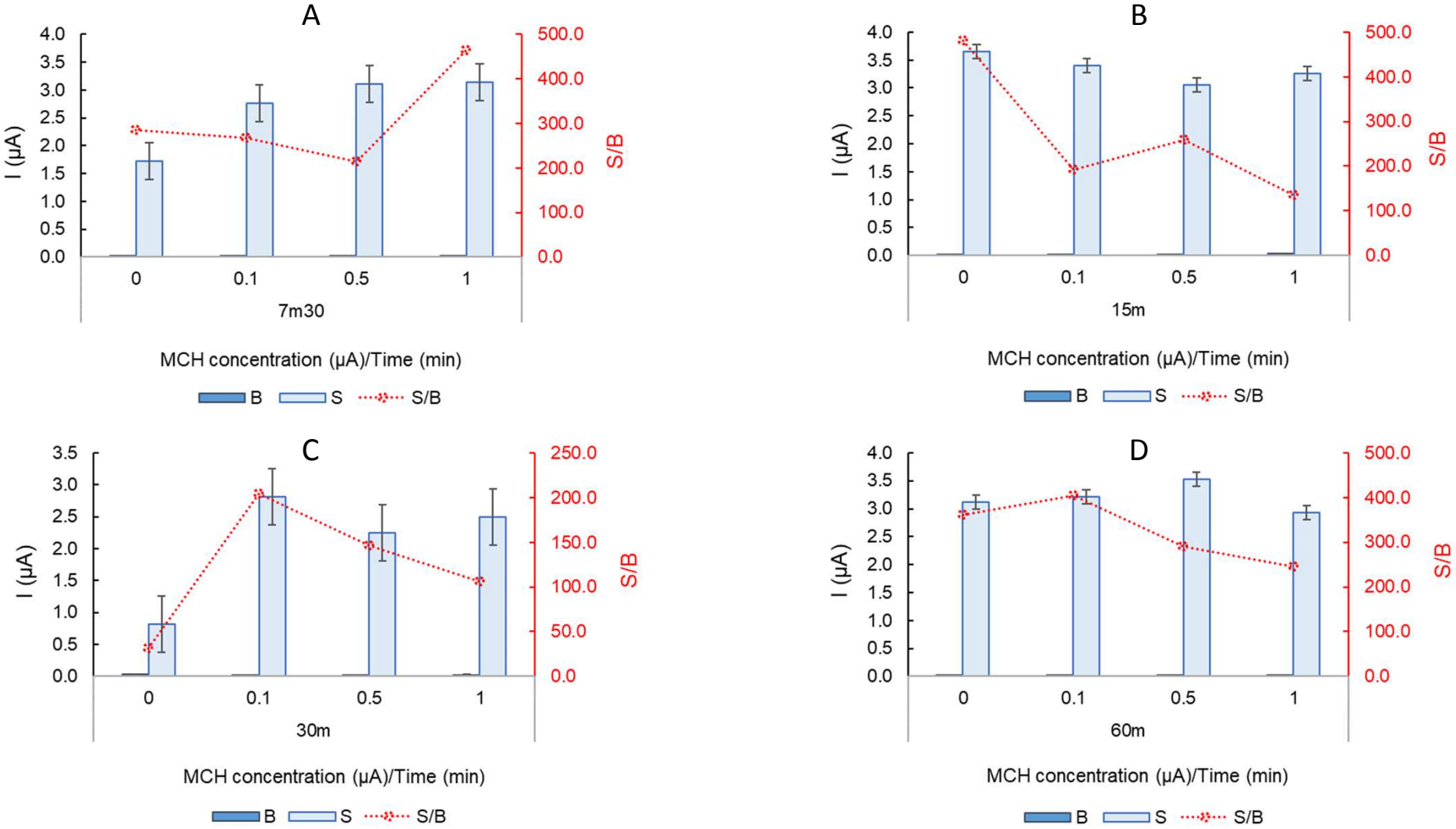

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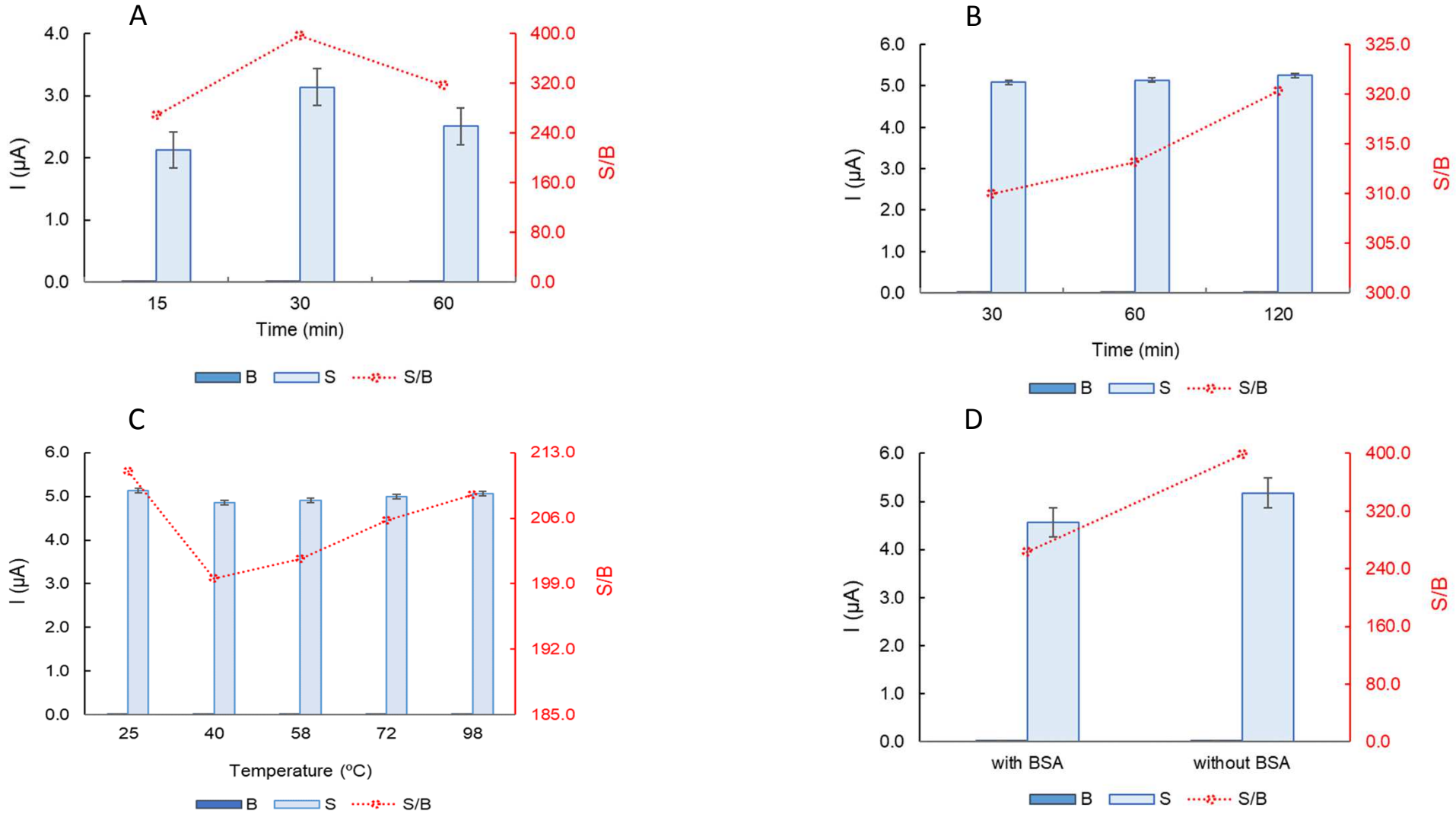

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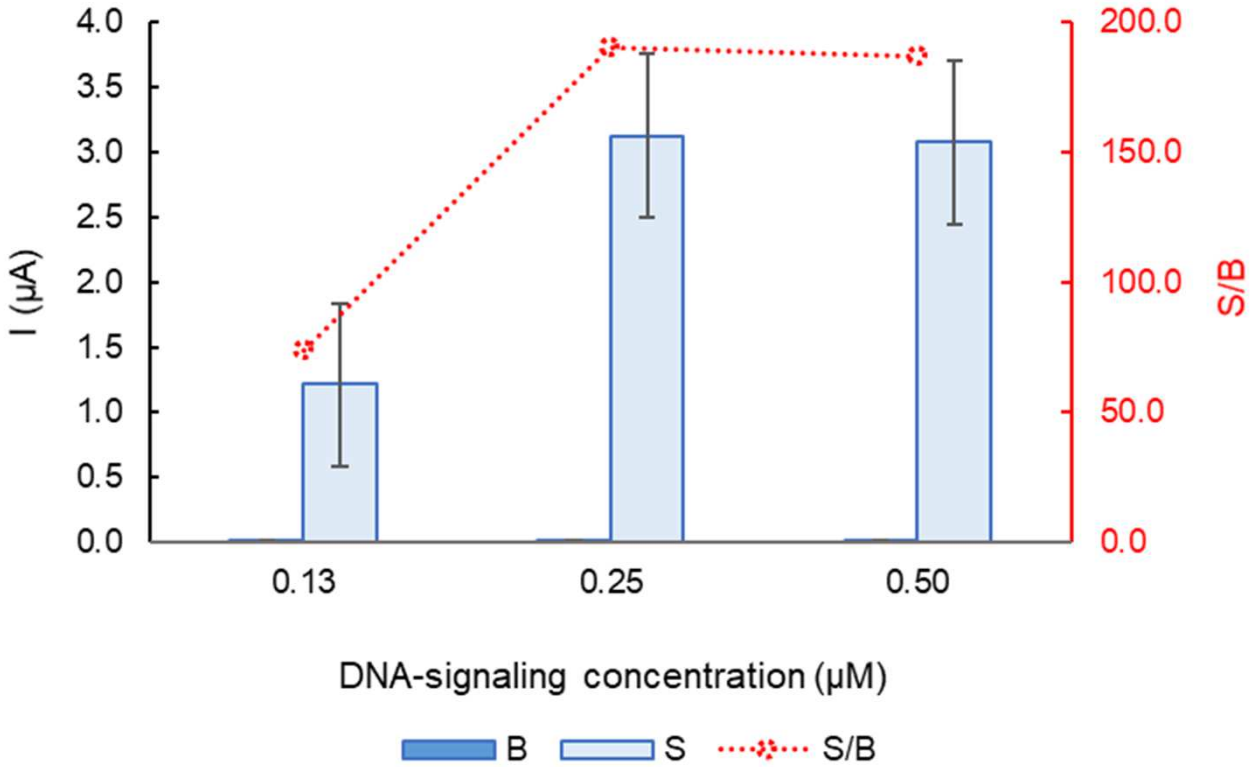

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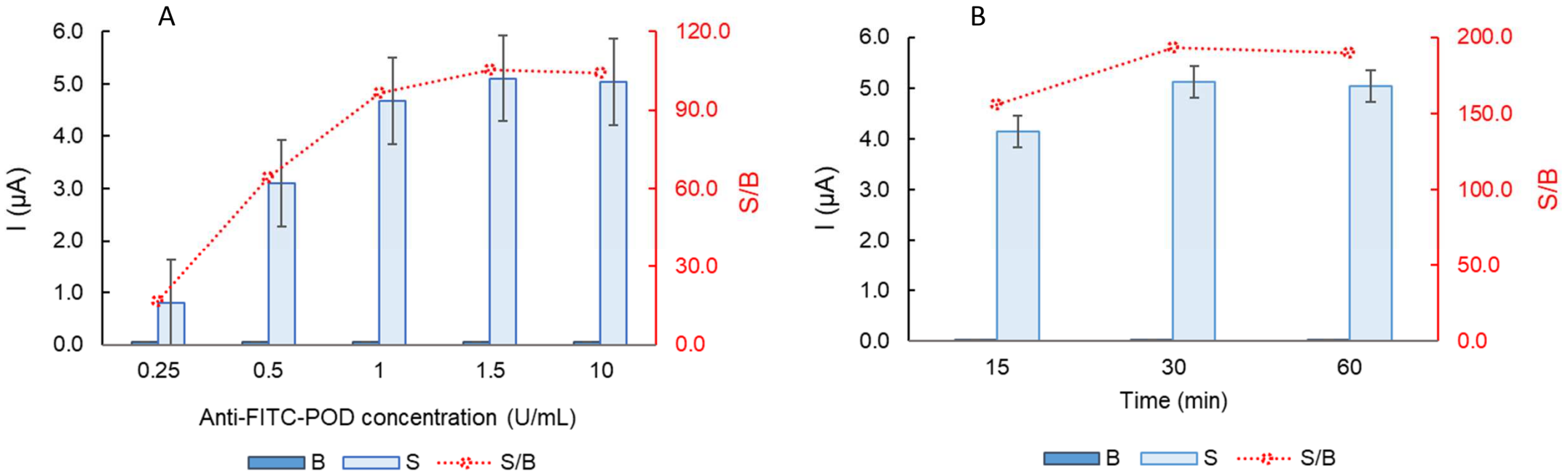

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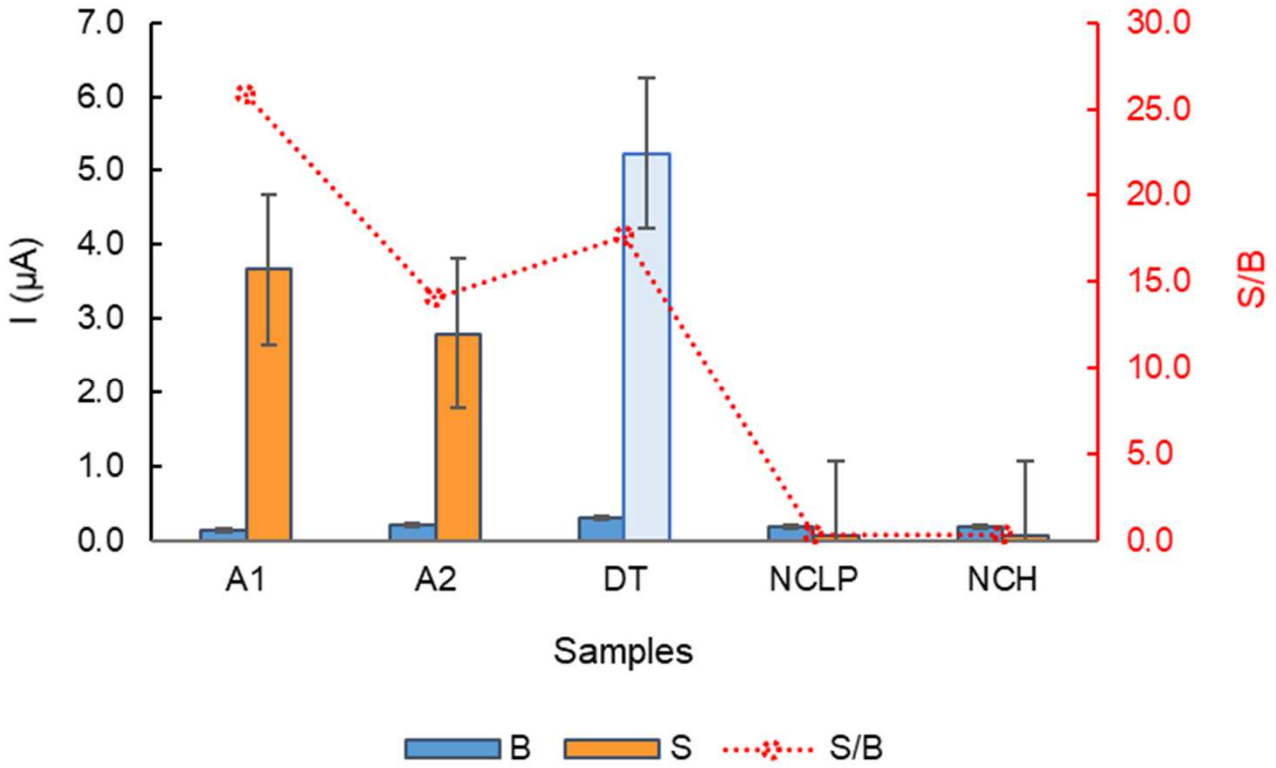

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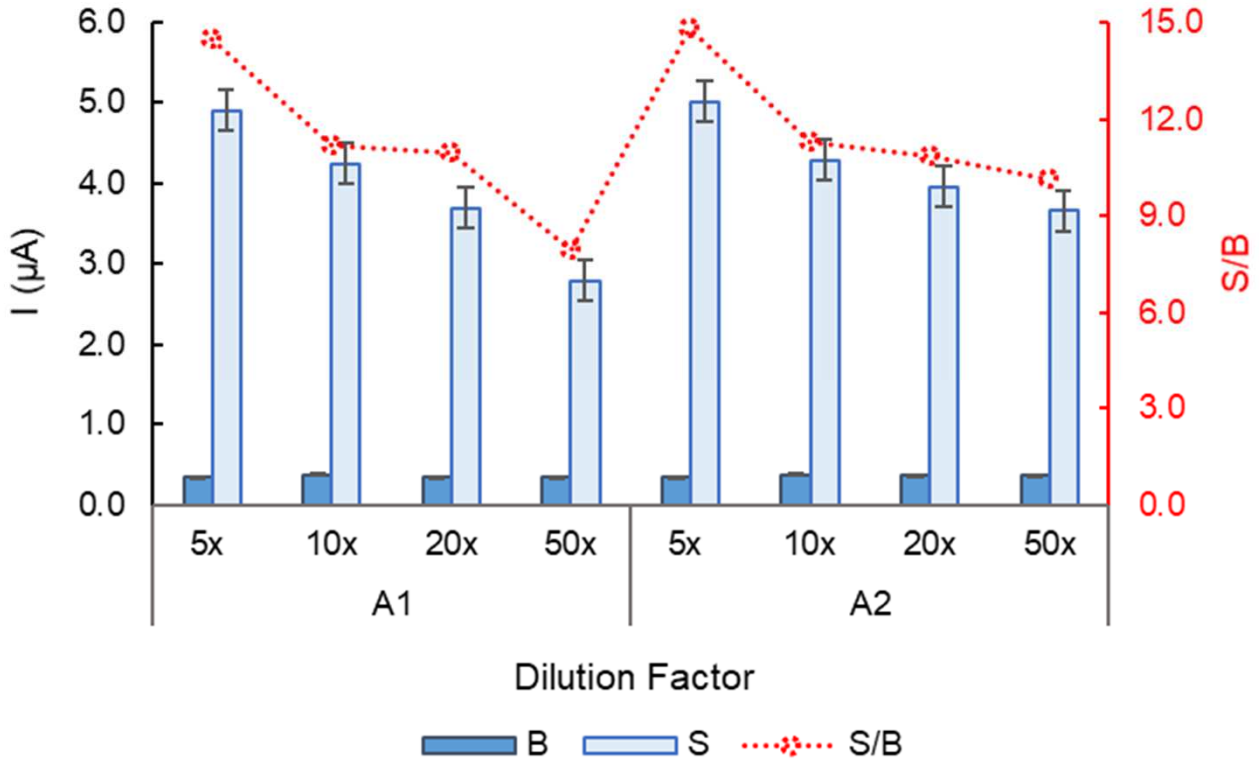

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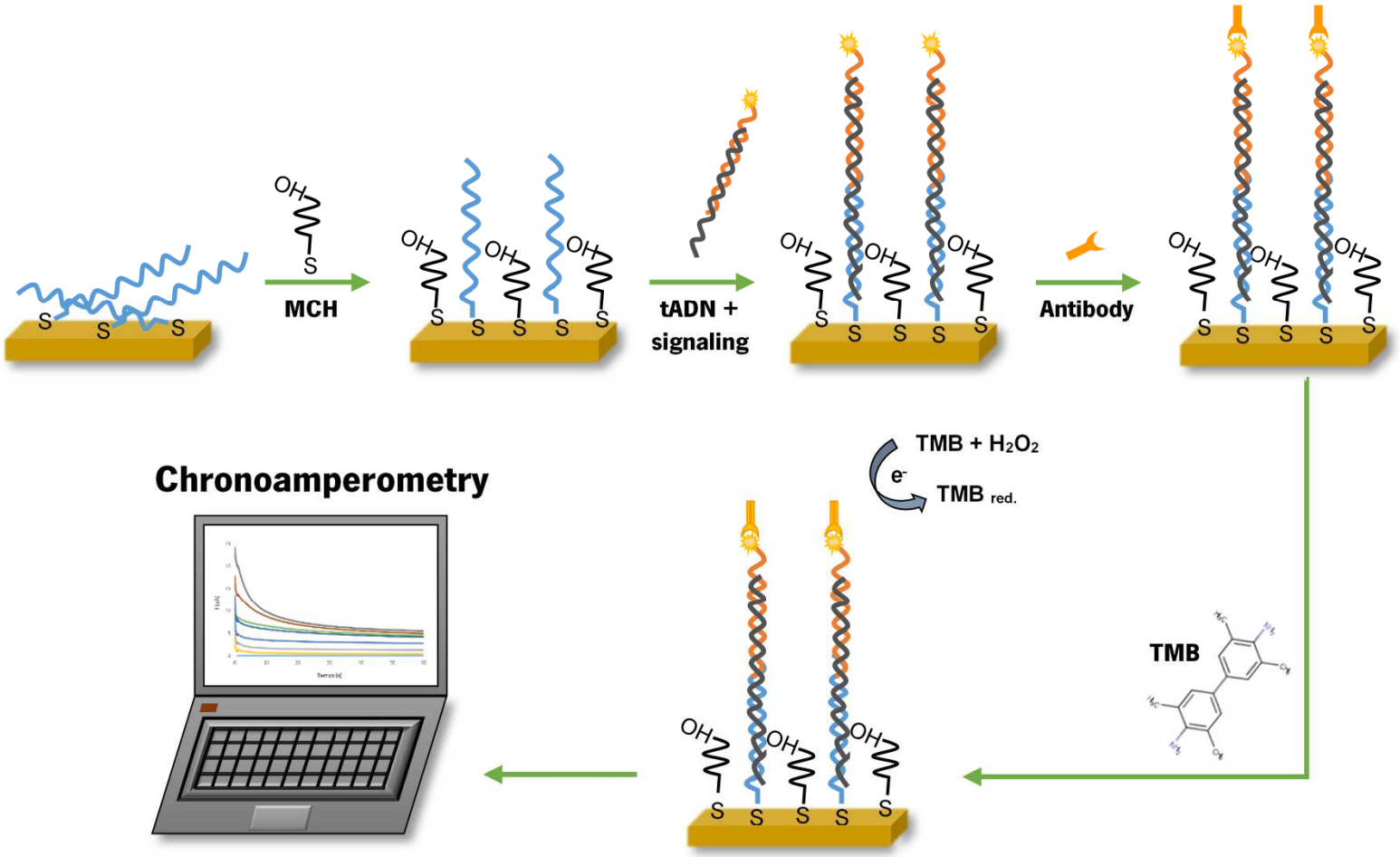
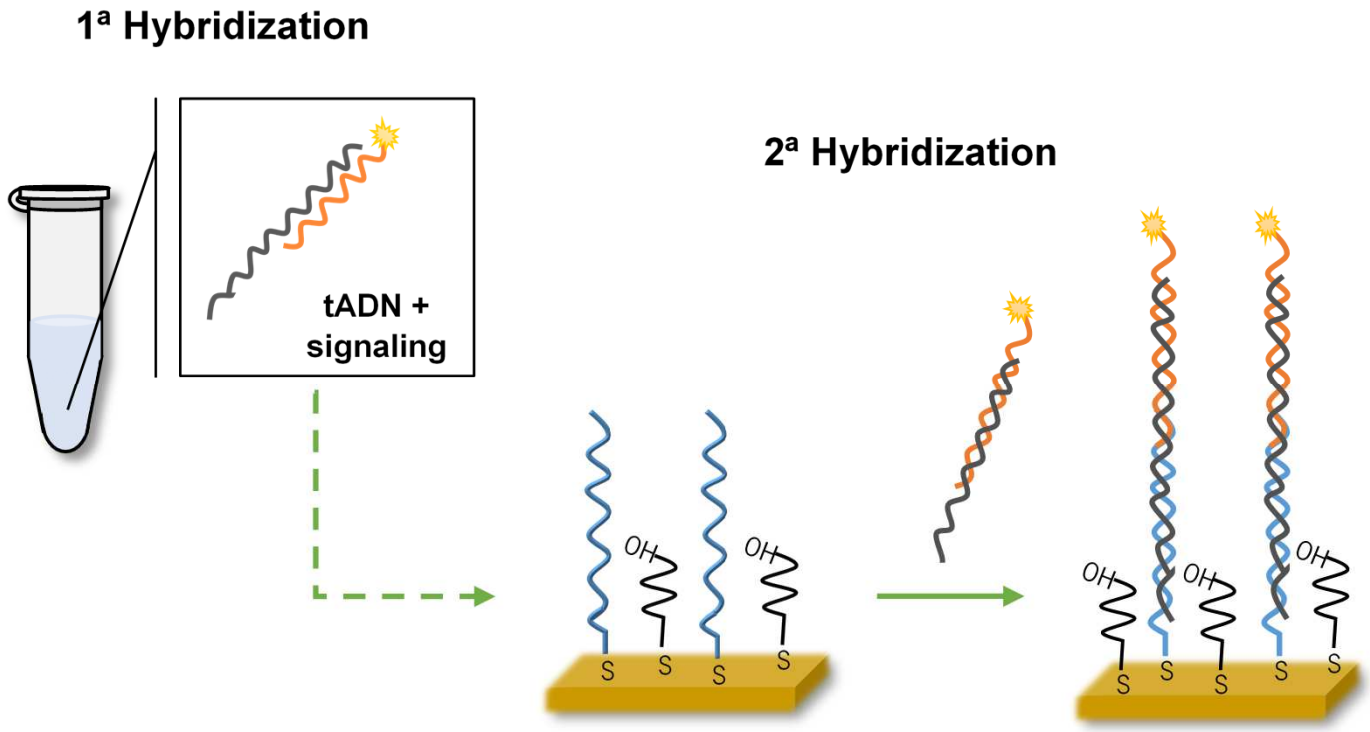


Scheme 1 - General procedure for the development of an electrochemical genosensor.



Scheme 2 - General scheme of the sandwich format assay. DNA-Capture, DNA target, DNA-signaling and MCH probes represented in light blue, grey, orange and black, respectably. The fluorescein protein is represented in yellow.

Highlights

- PCR-free electrochemical biosensing of toxic dinoflagellate nucleic acids.
- Electrochemical disposable genosensor for targeting Alexandrium minutum coding sequence.
- LOD of 25 pM (0.6 fmol in 25 µL).
- Unequivocal detection of A. minutum in Atlantic Ocean water.

Declaration of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Credit Author Statement

Stephanie Morais: Investigation, Writing - Original Draft, Visualization
Piedade Barros: Validation, Resources, Writing - Review & Editing, Visualization, Project administration
Marlene Santos: Validation, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration
Cristina Delerue-Matos: Validation, Resources, Funding acquisition, Writing - Review & Editing, Visualization
Andreia C. Gomes: Conceptualization, Validation, Writing - Review & Editing, Visualization, Supervision
Fátima Barroso: Conceptualization, Resources, Validation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration