Biosensors and Bioelectronics 134 (2019) 76–82

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Electrochemical genosensor for the direct detection of tailed PCR amplicons incorporating ferrocene labelled dATP

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ARTICLE INFO

Keywords:
Karlodinium armiger
PCR
Square wave voltammetry
Redox labelled nucleotides
Ferrocene
Tailed primers

ABSTRACT

An electrochemical genosensor for the detection and quantification of *Karlodinium armiger* is presented. The genosensor exploits tailed primers and ferrocene labelled dATP analogue to produce PCR products that can be directly hybridised on a gold electrode array and quantitatively measured using square wave voltammetry. Tailed primers consist of a sequence specific for the target, followed by a carbon spacer and a sequence specifically designed not to bind to genomic DNA, resulting in a duplex flanked by single stranded binding primers. The incorporation of the 7-(ferrocenylethynyl)-7-deaza-2′-deoxyadenosine triphosphate was optimised in terms of a compromise between maximum PCR efficiency and the limit of detection and sensitivity attainable using electrochemical detection via hybridisation of the tailed, ferrocene labelled PCR product. A limit of detection of 277aM with a linear range from 315aM to 10 fM starting DNA concentration and a sensitivity of 122 nA decade^{-1} was achieved. The system was successfully applied to the detection of genomic DNA in real seawater samples.

1. Introduction

Karlodinium is a dinoflagelate genus involved in harmful algal blooms (HAB) that produces huge fish killing-events around the world including areas in South Africa, Europe, North America, and Australia with impact in aquaculture ponds and local wild fauna (Fernandez et al., 2006). Identification and quantification of *karlodinium* species can be used as an early warning tool for the initiation of mitigation strategies to prevent HABs, including moving fish cages from the path of the algae bloom, or targeting the bloom with chemicals or other biological control agents (Fernández-Tejedor et al., 2007). Current monitoring programs rely on light microscopy to perform algae identification and counting. However, microscopy is inherently slow, laboratory based and prone to misidentification with other dinoflagelates such as *Alexandrium* genus (Figueroa et al., 2010) due to the poor morphology features of *Karlodinium* genus. Furthermore, it is impossible to distinguish between *Karlodinium* species, which is very important as their rate of proliferation and toxicity varies from one to another. Since 1994, recurrent fish killing events have been reported in Spain's Alfacs Bay, which were attributed to the proliferation of two co-existing *Karlodinium* species: *K. veneficum* and *K. armiger*. Both have been reported to be mixotrophic and combine photosynthesis with prey feeding but with notable differences (Berge et al., 2012). Whereas *K. veneficum* relies more on photosynthesis, *K. armiger* growth rate is strongly dependant on prey feeding. In fact, *K. armiger* shows a pronounced swarming behaviour, with the ability to form a feeding aggregate allowing ingestion of prey several times larger than itself, including larvae of commercially important bivalves and finfish, and as a consequence *K. armiger* represents a threat for different trophic levels and is also more toxic than *K. veneficum* (Berge et al., 2012).

To distinguish between *K. armiger* and *K. veneficum* a chloroplast counting per cell can be implemented but this method is extremely time consuming limiting its use in monitoring programs (Fernandez et al., 2006). Flow cytometry, a high throughput analytical tool that allows the discrimination and quantification of cells, whilst also providing information regarding the life cycle stages of dinoflagellates, is another

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https://doi.org/10.1016/j.bios.2019.03.060
Received 2 January 2019; Received in revised form 26 March 2019; Accepted 27 March 2019
Available online 30 March 2019
0956-5663/

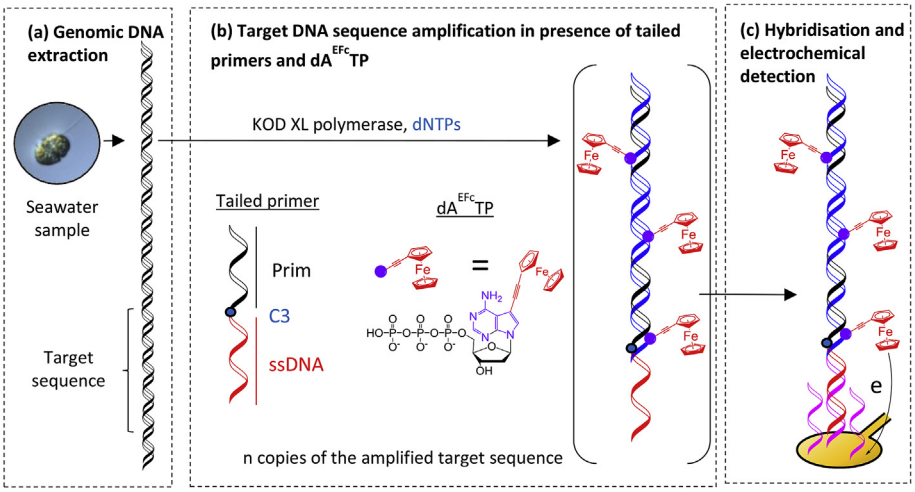


Fig. 1. Schematic of the assay (a) Extraction of *K. armiger* genomic DNA from seawater; (b) DNA is amplified by PCR using KOD XL polymerase in the presence of tailed primers and ferrocene labelled dATP (**dA^{EFc}TP**); (c) Redox labelled PCR product hybridises to a short capture probe complementary to the tail sequence, which is immobilised on the surface of individual gold electrodes of a microfabricated array. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

alternative. However, it is more suited to mono-specific blooms of one or two species and the instrumentation required is expensive and complex (Figueroa et al., 2010). Molecular tools such as qPCR has also been employed for the identification and quantification of different algae species, such as that developed to detect and quantify *K. armiger* and *K. veneficum* in seawater samples (Toldrà et al., 2018), and whilst it is specific, robust and relatively rapid, it is quite expensive and requires trained personnel and infrastructure.

Here, we present a new generic approach for use in electrochemical genosensing exploiting a combination of tailed primers and redox labelled dNTPs (Hocek and Fojta, 2011), using the detection of *K. armiger* in seawater samples as a model system. The use of tailed primers in both the PCR and isothermal amplification has previously been reported (Jauset-Rubio et al., 2016a, 2016b), and also the electrochemical detection of PCR products using the ferrocene labelled dATP analogue (7-(ferrocenylethynyl)-7-deaza-2′-deoxyadenosine 5′-triphosphate, **dA^{EFc}TP**) (Brázdilová et al., 2007), but this is the first demonstration of their combination and direct detection of the PCR product on microfabricated gold electrode arrays (Fig. 1).

The vast majority of reports detailing DNA biosensors require the generation of post-amplification generation of single-stranded DNA for its subsequent hybridisation to a surface-immobilised probe (Junhui et al., 1997), and this is often followed by another hybridisation with a labelled reporter probe, to generate a signal proportional to the quantity of DNA.

Among the different transduction options, electrochemical transduction have some advantages over other techniques, including high sensitivity, compatibility with microfabrication and miniaturization, simple and inexpensive instrumentation required, with an inherent low cost and power consumption as well as its tolerance to sample turbidity (Wang, 1999). The electrochemical detection of DNA hybridisation is mainly based on the electrochemical detection of labels (organic dyes, metal complexes, enzymes or metal particles) that intercalate within the double stranded DNA, are electrostatically adsorbed on the dsDNA phosphate backbone or hybridise to the target through a secondary labelled reporting probe (Kerman et al., 2004).

In our approach we overcome the need to generate single stranded DNA for detection via hybridisation to an immobilised probe exploiting a specifically designed tailed forward primer, which consist of a single stranded DNA sequence (“tail”) that is added to the 5′-end of the primer using a 3-C alkyl chain spacer, preventing elongation of the tail during amplification. This results in a PCR product that is a duplex of the targeted sequence tethered with a single stranded tail that hybridises with a surface immobilised probe (Fig. 1b).

To avoid the use of labels that require to be added in a further step following hybridisation, an alternative is to totally or partially replace the natural dNTPs employed in PCR with modified dNTPs tethered with electrochemically active moieties such as, for example, ferrocene (Brázdilová et al., 2007), anthraquinone (Balintová et al., 2011), or benzofurazane (Balintová et al., 2013). We chose to use the ferrocene labelled dATP analogue, as ferrocene is a reversible and stable label, its oxidation peak when incorporated into DNA is within the gold electrode potential window (450 mV vs Ag/AgCl 3 M KCl reference electrode) and the **dA^{EFc}TP** has been demonstrated to be a good substrate for several DNA polymerases such as Klenow (exo) or DyNAzyme (Brázdilová et al., 2007).

2. Materials and methods

2.1. Reagents

Synthetic oligonucleotides were purchased from Biomers (Ulm, Germany), KOD XL polymerase was purchased from Merck illipore (Madrid, Spain), the SYBR Green dye was purchased from Applied Biosystems (Spain), HRP substrate formulation tetramethylbenzidine (TMB) enhanced one component HRP membrane was purchased from Diarect AG (Germany), GelRed™ Nucleic Acid Gel Stain from Biotium (Barcelona, Spain) and the certified molecular biology agarose gel powder from Bio-Rad Laboratories S.A. (Barcelona, Spain). Three-millimetre thick polymethylmethacrylate (PMMA) was purchased from La Indústria de la Goma (Tarragona, Spain) and double-sided medical grade adhesive foil ARSeal 90880 from Adhesive Research (Ireland). All other chemicals were obtained from Sigma-Aldrich S.A. (Barcelona, Spain) and used as received. Natural dNTPs were purchased from ThermoFischer Scientific (Barcelona, Spain) and dA^{EFc}TP was synthesised following the Sonogashira reaction (Brázdilová et al., 2007).

DNAse free water from Fisher Bioreagents was used to prepare all reagents involved in PCR and the rest of the solutions were prepared using high purity deionised water (18 MΩ) produced with a Milli-Q RG system (Millipore Ibérica, Spain).

2.2. Oligonucleotide sequences

Primers used were designed to be specific for *K. armiger* target (Toldrà et al., 2018) and avoid cross-reactivity with other related toxic microalgae such as *K. veneficum* and the tails in the tailed primers were designed to avoid cross-reactivity between them and also with the primers. (see Table 1).

2.3. DNA extraction from samples

Seawater samples were spiked with cultured *K. armiger* and *K.*

Table 1
List of oligonucleotide sequences and their respective modifications (underlined).

Oligo	Sequence
Karlo FwP T	5′-ATT ACG ACG AAC TCA ATG AA – C3 – ATA GCT TCA CAG CAG AGG TTA CAA C-3′
Karlo RvP	5′-ACA CAC ATC CAA CCA TYT CAC TG-3′
Karlo RvP T	5′-TGT AAA ACG ACG GCC AGT – C3 – ACA CAC ATC CAA CCA TYT CAC TG-3′
KA target	5′-ATA GCT TCA CAG CAG AGG TTA CAA CAC CAA TGC TGC TCC GCT ACC CGC GAT CTC ATG CAC CAG GGA GCG GCA AGA AGC CAG AGC TTC AAG ACA CCC CTA CCC CCG TGC AGG AGC TCA CAA AGA AAG TTC ACA GTG AGA TGG TTG GAT GTG TGT-3′
KA SP	5′- TTC ATT GAG TTC GTC GTA ATT TTT TTT TTT TTT TT-3′-C6-SH
NS SP	5′- GTC GTG ACT GGG AAA ACT TTT TTT TTT TTT - 3′-C6-SH
Reporter probe-HRP	HRP-5′-ACA CAC ATC CAA CCA TYT CAC TG-3′

Y represent the wobble C + T.
[Note: Whilst in the final format of the assay using the ferrocene labelled dNTPs only tailed forward primer would be used, for developmental work both the reverse and forward primers were tailed. This renders a double tailed amplicon, with one tail for hybridising to the surface immobilised probe, and the other tail to hybridise to a horse radish peroxidase labelled probe. This was simply used to confirm that amplification with the tailed primers and hybridisation of the PCR amplicon was successful.].

veneficum cells at two different concentration levels (**Alert level** ≈ 200,000 cells/L and **Mortality level** ≈ 1,000,000 cells/L). (Fig. 5b). Genomic DNA was extracted using the Biomeme (BIM) isolation kit (Biomeme, Philadelphia, USA) following a protocol previously developed (Toldrà et al., 2018). Briefly, 50 mL of sample is centrifuged at 3700 g for 25min to harvest all cells in suspension. Then, the pellet is re-suspended in 300 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), transferred to a 2 ml cryotube that contains ~50 µg of 0.5 mm diameter zirconium glass beads (Biospec, USA) and lysed using a BeadBeater-8 (BioSpec, USA) pulsed for 45s at full speed. Subsequently, 250 µL of the lysed sample is pumped through an ion-exchange cartridge coupled to a syringe up and down 10 times (10 pumps) to capture all the DNA. Then, the ion cartridge is washed with 500 µL of Biomeme Protein Wash solution (1 pump), then with 750 µL of Biomeme Wash Buffer (1 pump) and finally the genomic DNA is eluted with 500 µL of Biomeme Elution buffer (5 pumps).

2.4. qPCR

The qPCR assay was performed using an ABI 7300 thermocycler (Applied Biosystems, Thermo Fisher Scientific, Spain) with previously optimised parameters (Toldrà et al., 2018). Briefly, a two-step cycling protocol was carried out, with an initial denaturation step at 95 °C for 10 min, followed by 45 cycles at 95 °C for 20 s and 58 °C for 30 s. Reaction mixture contained 10 µL SYBR Green dye 2x, 2 µL of primers (Karlo FwP T and Karlo RvP) at 5 µM concentration stock each, 2 µL of DNA extracts or calibration curve patrons made by serial solution of DNA target and 2 µL of DNAse free water.

2.5. PCR

The PCR was performed with a T100 thermal cycler (Biorad) using the following cycling protocol: 95 °C for 2 min, followed by 45 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by a final elongation step at 72 °C for 5 min. Each 10 µL reaction mixture contained 0.1 units of KODXL and KODXL buffer 1X, both forward and reverse primers (Karlo FwP T and Karlo RvP) at 100 µM, dGTP, dCTP, dTTP at 200 µM and different ratios **dA^{EFc}TP**:dATP whilst maintaining [**dA^{EFc}TP** + dATP] = 200 µM as final concentration. To check DNA hybridisation on the electrodes, Karlo RvP was replaced by Karlo RvP T in the PCR mixture.

2.6. Agarose gel electrophoresis

Amplification products were checked by agarose gel electrophoresis. The gel was made with ultralow pure agarose (3% w/v) in 1 × Tris-Borate- EDTA buffer (TBE) and stained with GelRed™nucleic acid stain. A mixture of 2 µL of PCR product with 4 µL of loading buffer 2x was loaded per gel well, electrophoresis was performed at 100 V for 22 min and gels were visualized in a UV transilluminator at λ = 254 nm.

2.7. Electrode fabrication

The electrode array was designed with a set of nine circular working electrodes (1 mm^2) and a rectangular counter electrode (4 mm^2). It was fabricated by sputtering on a 75 × 25mm soda-lime glass slides substrate (Sigma-Aldrich, Spain) as described previously with minor modifications (del Río et al., 2016). Briefly, a positive photoresist AZ1505 (MicroChemicals GmbH, Germany) was deposited by spin coating at 4000 rpm for 30sec on a pre-cleaned and dried glass slide. Then, the photoresist was exposed to UV light for 4 s using a chromium mask in contact mode (LED Paffrath GmbH, Rose FotoMasken, Germany) and the transferred pattern was developed using the commercial developer AZ726. Following development, the glass slide was introduced into the sputtering chamber (ATC Orion 8-HV, AJA International Inc., USA) and was subjected to an oxygen plasma etching using AC O_2/Ar (5 $cm^3{\cdot}s^{-1}$ of Ar, 5 $cm^3{\cdot}s^{-1}$ of O_2, 50 W) for 5 min. Then, a layer of 30 nm of Ti/TiO_2 was sputtered (oxygen flow rate: 5 $cm^3{\cdot}s^{-1}$ of O_2 for the first 10 nm, then increase up to 20 $cm^3{\cdot}s^{-1}$ for the last 5 nm. Ar flow rate: constant 5 $cm^3{\cdot}s^{-1}$). Last step consisted in the deposition of 100 nm of Au by AC sputtering (5 $cm^3{\cdot}s^{-1}$ of Ar, 5 $cm^3{\cdot}s^{-1}$ of O_2, 50 W). Lift-off was done by sonication during 5 min in acetone, then 5 min in isopropanol and finally rinsed with Milli-Q water.

2.8. Electrode functionalisation with capture probe

Electrodes were cleaned with soap prior to modification, then rinsed with milli-Q water and dried with N_2. One microlitre of a solution containing 1 µM surface probe (KA SP or NS SP), 100 µM mercaptohexanol in 1 M KH_2PO_4 was dropcasted per each electrode and the array was incubated at room temperature (22 °C), for at least 16 h in a humidity saturated chamber. After incubation, electrodes were rinsed with Milli-Q and dried with N_2.

2.9. Microfluidic fabrication and mounting

Microfluidics were fabricated using double adhesive gasket (Adhesive Research, Ireland) and 2 mm thickness PMMA cover plates patterned using a CO_2 laser marker (Fenix, Synrad, USA). Following electrode array functionalisation, double adhesive gasket and PMMA were aligned and bonded by pressure to produce a 7 µL microfluidic chamber where DNA hybridisation and electrochemical measurements were carried out (Fig. 2). Microfluidic chambers were washed with 200 µL of PBS Tween-20, 200 µL of Milli-Q and dried with N_2. Functionalised arrays, housed within microfluidics are stable for at least one week stored at 4 °C.

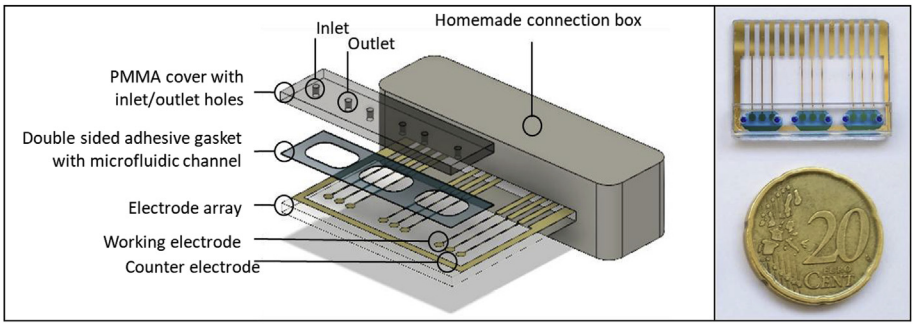


Fig. 2. Schematic representation and actual picture of the electrode array housed within the microfluidic cell. Electrode array, double adhesive gasket and PMMA cover are bound together to create 7 µL cells where hybridisation and electrochemical measurements are carried out. Electrode is connected to the potentiostat trough a homemade connection box.

2.10. Amplicon hybridisation on electrode arrays

Following amplification, 7 µL of PCR product was directly incubated in the microfluidic chamber for 30 min at room temperature (22 °C) in a humidity saturated chamber. The microfluidic chamber was then washed 3 times with 200 µL of PBS Tween-20 and 200 µL of PBS containing $Ru[NH_3]_6Cl_3$ (Ruthenium(III) hexamine) 0.1 mM. Square wave voltammetry was subsequently performed by placing a Ag/AgCl (1 M KCl) reference electrode on the droplet of PBS containing ruthenium (III) hexamine formed on top of the PMMA block between the inlet and the outlet of the microfluidic cell to be measured.

2.11. Secondary hybridisation with reporting probe (only used as control to check DNA hybridisation)

After amplicon hybridisation and square wave voltammetry measurements, microfluidic chambers were then flushed with 200 µL of PBS and incubated with 7 µL of 10 nM reporter probe-HRP in PBS for 30 min at room temperature (22 °C) in a humidity saturated chamber. Microfluidic chambers were then flushed 3 times with 200 µL of PBS Tween-20 and 200 µL of PBS and subsequently the microfluidic chambers were filled with 100 µL of TMB enhanced one component HRP membrane and allowed to react for 5 min. Finally, fast chronoamperometric measurements were carried out placing the Ag/AgCl (1 M KCl) reference electrode, as explained above.

2.12. Electrochemical measurements

All electrochemical measurements were performed with a potentiostat/galvanostat PBSTAT 12 Autolab controlled with Nova 2.0 Software. Electrode array was connected to the potentiostat through a homemade connector and electrochemical measurement were done using an external Ag/AgCl reference electrode, and the internal gold working and counter electrodes (Fig. 2).

Square wave voltammetry measurements were carried out following amplicon hybridisation on electrode arrays in PBS containing Ruthenium(III) hexamine (0.1 mM) by applying a potential from 0.2 V to 0.5 V with a 5 mV step, 25 mV modulation amplitude and 50 Hz frequency.

Fast chronoamperometric measurements were carried out after a secondary hybridisation with the reporting probe by applying a potential of −0.2 V vs Ag/AgCl RE for 0.5 s and then reading the current output.

Results are represented as the mean value ± standard deviation for triplicate measurements.

3. Results and discussion

3.1. Direct DNA hybridisation after PCR amplification and electrochemical measurements

Fig. 3 illustrates the experiments carried out to demonstrate the concept of the method presented here. To prove that tailed primers can be used to capture PCR products specifically on a gold electrode, and that the incorporated ferrocene could be measured, PCR was carried out using tailed forward and reverse primers [**Note:** Reverse primer with tail is only used as a control in this experiment but will not be used in the final method].

One electrode was modified with a thiolated non-specific surface probe (NS SP) (electrode A) and two electrodes with a thiolated specific surface probe (KA SP) (electrodes B and C). Subsequently, electrodes A and B were incubated with PCR product that was amplified in the presence of tailed primers, 100% **dA^{EFc}TP** and 10 nM initial DNA target concentration (Fig. 3a). After the excess of PCR product was removed by washing, square wave voltammogram was recorded in the presence of PBS and ruthenium (III) hexamine (Das et al., 2018). An oxidation peak at 450 mV with a peak intensity of 100 ± 5 nA was only observed at the electrode modified with the specific surface probe shown (Fig. 3b). To determine that the oxidation peak was coming from ferrocene and not from the DNA itself, electrode C was incubated with an amplicon produced by PCR in the presence of tailed primers and natural primers but not containing **dA^{EFc}TP** and no oxidation peak was observed, further confirming that the signal was specifically coming from the ferrocene incorporated into the DNA (Fig. 3a).

To confirm that amplicons were captured on the gold electrodes A, B and C, all electrodes were exposed to a reporter probe-HRP and chronoamperometry was performed in the presence of precipitating TMB (Fig. 3c). As expected, amperometric signals were observed at Electrode B and C (−380 ± 12 nA and −736 ± 22 nA, respectively), with only negligible currents observed at Electrode A (−17 ± 6 nA) (Fig. 3d).

The higher current observed at Electrode C as compared to B can be explained by the higher amount of amplicon generated using natural dNTPs, as described below (Fig. 4a).

3.2. Effect of dA^{EFc}TP:dATP ratio on PCR amplification yield and electrochemical sensitivity

Although **dA^{EFc}TP** is a good substrate for DNA polymerases (Brázdilová et al., 2007), similar to other related 7-alkynyl or 7-aryl-7-deazapurine dNTPs (Kielkowski et al., 2014), in PCR amplification the bulkier modified dNTPs often give lower yields due to the difficult reading of the polymerase through hypermodified templates. Therefore, once the incorporation and electrochemical detection of the modified dNTPs had been demonstrated using 100% **dA^{EFc}TP** in the PCR mixture, mixtures of **dA^{EFc}TP** with natural dATP in different ratios (100%, 80%, 60%, 40% and 20%) were evaluated in terms of PCR yield and electrochemical signal detected.

Calibration curves were performed at different starting concentrations of DNA target, ranging from 10 nM to 1 fM for each **dA^{EFc}TP**:dATP ratio. It is known that modified dNTPs decrease polymerase activity, leading to a decreased amplification efficiency (Wlassoff and King, 2002), and as can be seen in Fig. 4a, decreasing the **dA^{EFc}TP**:dATP ratio does indeed improve the limit of detection. This effect can be exploited

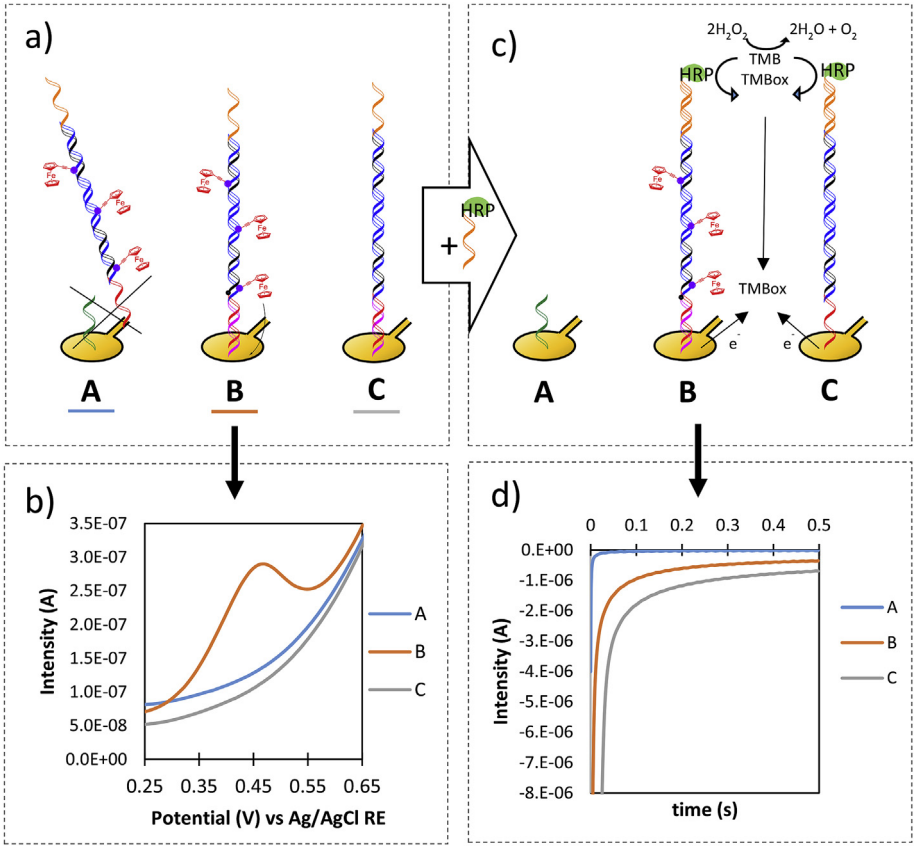


Fig. 3. Schematic of demonstration of proof-of-concept of detection of double tailed PCR amplicons incorporating ferrocene labelled dNTPs as detailed in the text.

to tune the linear range and the limit of detection of the method according to the specific needs of the assay.

To ensure that the **dA^{EFc}TP** is still incorporated using the different **dA^{EFc}TP**:dATP ratios, PCR product from a 10 nM starting concentration of target, were hybridised to the gold electrode immobilised probes and detected using SWV. As can be observed in Fig. 4b, **dA^{EFc}TP** is incorporated in all the ratios tested but the peak intensity depends on two factors - the amplification yield and the ratio **dA^{EFc}TP**:dATP used. For saturated PCR reactions (20–60%, which show the same band intensity on the gel), the lower ratio results in a decreased ferrocene peak intensity, attributable to a reduced incorporation of the modified dATP into the amplicon with the lower **dA^{EFc}TP**:dATP ratios. The peak intensity dependence on the number of **dA^{EFc}TP** incorporated on the DNA was previously reported (Brázdilová et al., 2007). At the higher ratios of 80–100%, the PCR amplification yield is lower and thus less molecules of DNA are captured on the electrode, consequently less molecules of ferrocene would available to be measured and lower ferrocene peak intensities would be obtained.

In order to be able to detect the *K. armiger* in a relevant concentration range (10-1 fM, when *K. armiger* population starts to be dangerous) the **dA^{EFc}TP/dATP** ratio has to be between 20 and 40%. We chose 30% as the best ratio in terms of LOD and sensitivity because it is enough to reach 1 fM and provides more sensitivity than 20% (data not shown).

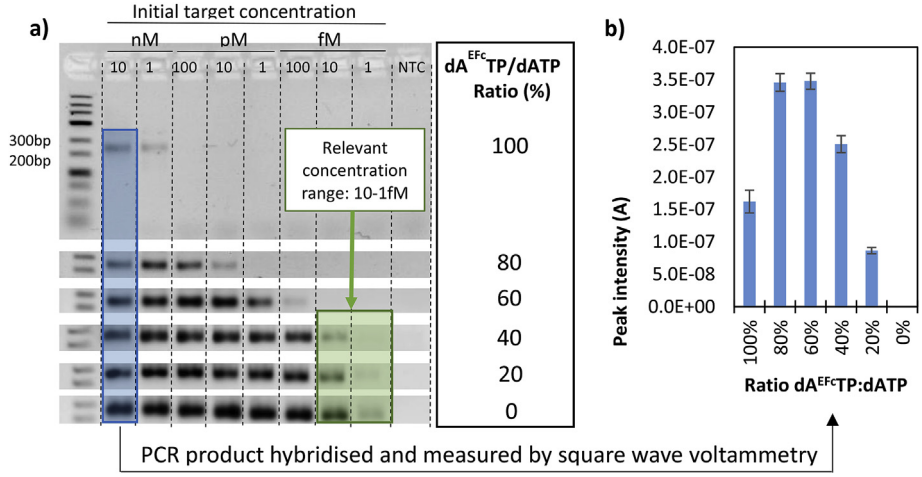


Fig. 4. (a) Agarose gel obtained for calibration curves using different **dA^{EFc}TP**:dATP ratios; (b) PCR products obtained from a 10 nM starting DNA concentration using different **dA^{EFc}TP**:dATP ratios were hybridised and measured by square wave voltammetry.

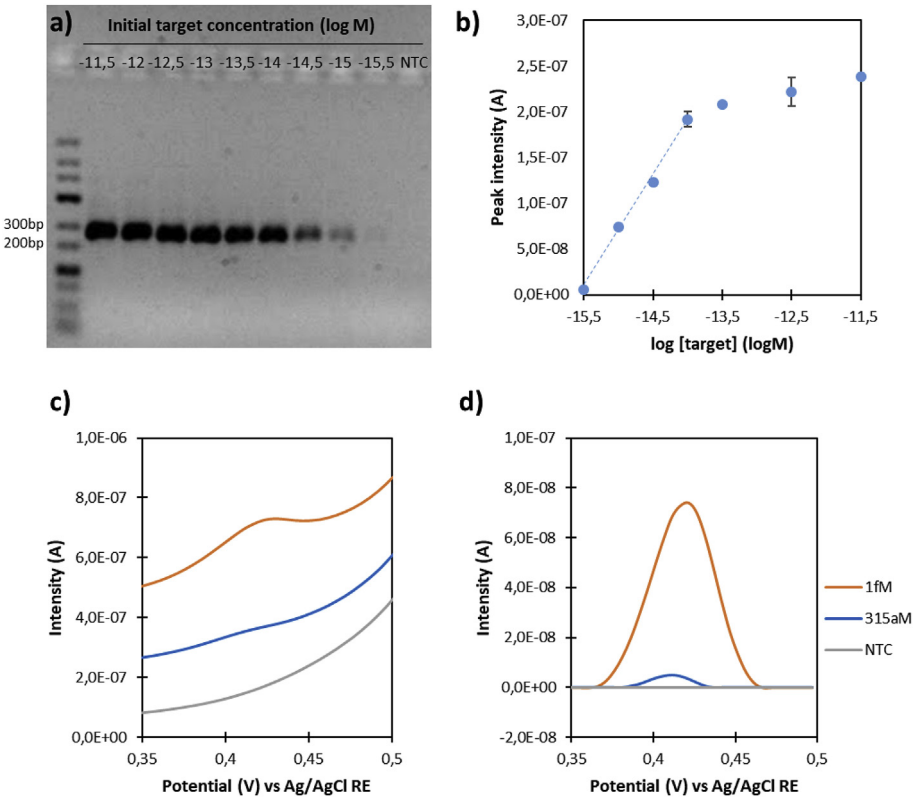


Fig. 5. (a) Agarose gel obtained for calibration curve using 30% **dA^{EFc}TP**:dATP ratio; and (b) electrochemical signal obtained for each concentration of the calibration curve. (c and d) are examples of the raw data voltammograms obtained for the non-template control (NTC), 315aM and 1 fM target concentration before and after baseline correction, respectively. Detection of *K. armiger* genomic DNA in seawater samples.

3.3. Calibration curve

A calibration curve was constructed over a wide range of concentrations (315 aM–3.15 pM (from −15.5 to 11.5 log M) using a 30% **dA^{EFc}TP**:dATP ratio (Refer to supplementary information, Fig. S1 for, Raw data voltammograms). Gel electrophoresis was carried out with the PCR products (Fig. 4i), and the resulting electrochemical calibration curve can be seen in Fig. 4ii. The assay was completed within 2,5 h PCR amplification (100 min), hybridisation (30 min) and detection (2 min). The limit of detection (LOD) achieved, defined as the concentration of the analyte at three times the standard deviation of the mean blank signal, was 277aM. The sensitivity was 122 nA·decade^{-1} with a linear range of 1.5 orders of magnitude, from 10 fM to 315aM starting DNA concentration.

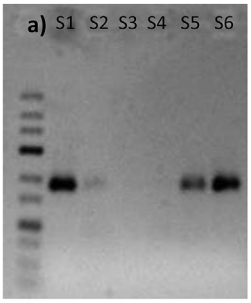
Seawater samples were spiked with cultured *K. armiger* and *K. veneficum* at two different levels of concentration, alert level (200,000 cells/L) and mortality level (1,000,000 cells/L). Genomic DNA was extracted and the concentrations estimated by the method developed in this work correlated very well with the concentrations estimated by the qPCR method developed previously (Toldrà et al., 2018). No cross-reactivity with *K. veneficum* was observed, or with any other organisms present in the seawater used in this work (Fig. 6). Please refer to the Supplementary Information, Fig. S2 for the raw data voltammograms.

3.4. Electrode array stability

The stability of functionalised electrodes was tested over 1 week. Electrode arrays were modified, functionalised, washed and dried with N_2 as described previously. Inlet and outlet holes for each microfluidic chamber were then sealed with adhesive gasket to insulate the electrodes, and the arrays were stored at 4 °C. Adhesive was removed and the microfluidic chamber washed as usual with 3 × 200 µL PBS-Tween and with 200 µL of Milli-Q and dried with N_2 prior to hybridisation with a tailed PCR product amplified with 30% dA^{EFc}TP:dATP ratio, from an initial target concentration of 10pM. In between measurements, the electrode was stored in the fridge at 4 °C. Fig. 7 shows there is no significant difference between the ferrocene signal obtained for the PCR product on day 1 and day 7, demonstrating the stability of the functionalised arrays over 1 week when they are stored, sealed in the fridge at 4 °C.

4. Conclusions

A novel and simple DNA electrochemical platform for the detection of genomic DNA after amplification by combining tailed primers and ferrocene labelled dATP analogue was demonstrated. As a proof of concept the method was applied to the detection of *K. armiger* genomic DNA in seawater samples. The assay was completed within 2.5 h and yielded a limit of detection of 277aM with a linear range from 315aM to 10 fM starting DNA concentration and a sensitivity of 122 nA·decade^{-1}. The developed assay eliminates the need for a single stranded DNA generation step prior to DNA hybridisation due to the use of tailed primers, and also avoids the need for any post hybridisation labelling for detection purposes due to the use of **dA^{EFc}TP**. The methodology is generic and ongoing work is focusing to extending to other genomic DNA targets in singleplex, duplex and multiplex formats, as well as replacing PCR with isothermal amplification, bringing the platform closer to deployment at the point-of-need.



b) Sample	Spiked cells	[target DNA] (fM) qPCR	[target DNA] (fM) present method	Recovery (%)
S1**	*K. armiger*	7.6	6.7	90
S2*	*K. armiger*	0.5	0.7	120
S3*	*K. veneficum*			
S4**	*K. veneficum*			
S5*	*K. armiger* + *K. veneficum*	1.3	1.3	100
S6**	*K. armiger* + *K. veneficum*	11	6.7	60

*Alert level: 200.000 cells/L
**Mortality level: 1.000.000 cells/L

Fig. 6. (a) Agarose gel electrophoresis obtained after PCR amplification of 6 seawater samples spiked with *K. armiger* and *K. veneficum*; (b) Sample composition and target DNA concentration estimated by qPCR and the method presented in this paper.

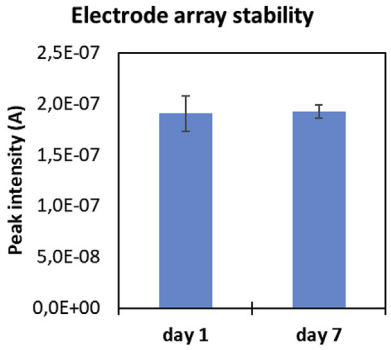


Fig. 7. Electrode array stability when stored at 4 °C ready to be used for hybridisation to PCR product and detection.

CRediT authorship contribution statement

Ivan Magriñá: Data curation, Investigation, Writing - original draft. **Anna Toldrà:** Data curation, Investigation, Writing - original draft. **Mònica Campàs:** Project administration, Investigation, Funding acquisition, Supervision, Writing - review & editing. **Mayreli Ortiz:** Data curation, Investigation, Writing - original draft. **Anna Simonova:** Data curation, Investigation, Writing - original draft. **Ioanis Katakis:** Project administration, Investigation, Funding acquisition, Supervision, Writing - review & editing. **Michal Hocek:** Project administration, Investigation, Funding acquisition, Supervision, Writing - review & editing. **Ciara K. O'Sullivan:** Project administration, Investigation, Funding acquisition, Supervision, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.03.060.

Funding

This work has been carried out with partial financial support from Spanish Ministerio de Economía y Competitividad for CIGUASENSING BIO2017-87946-C2-1-R and SEASENSING BIO2014-56024-C2-1. The work was also supported by the Czech Academy of Sciences (Praemium Academiae to M.H.), and by the European Regional Development Fund; OP RDE (No. CZ.02.1.01/0.0/0.0/16_019/0000729 to M.H.). The authors also acknowledge support from CERCA Programme/Generalitat de Catalunya.

References

Balintová, J., Plucnara, M., Vidláková, P., Pohl, R., Havran, L., Fojta, M., Hocek, M., 2013. Benzofurazane as a new redox label for electrochemical detection of DNA: towards multipotential redox coding of DNA bases. Chem. Eur J. 19, 12720–12731. https://doi.org/10.1002/chem.201301868.

Balintová, J., Pohl, R., Horáková, P., Vidláková, P., Havran, L., Fojta, M., Hocek, M., 2011. Anthraquinone as a redox label for DNA: synthesis, enzymatic incorporation, and electrochemistry of anthraquinone-modified nucleosides, nucleotides, and DNA. Chem. Eur J. 17, 14063–14073. https://doi.org/10.1002/chem.201101883.

Berge, T., Poulsen, L.K., Moldrup, M., Daugbjerg, N., Juel Hansen, P., 2012. Marine microalgae attack and feed on metazoans. ISME J. 6, 1926–1936. https://doi.org/10.1038/ismej.2012.29.

Brázdilová, P., Vrábel, M., Pohl, R., Pivoňková, H., Havran, L., Hocek, M., Fojta, M., 2007. Ferrocenylethynyl derivatives of nucleoside triphosphates: synthesis, incorporation, electrochemistry, and bioanalytical applications. Chem. Eur J. 13, 9527–9533. https://doi.org/10.1002/chem.200701249.

Das, J., Ivanov, I., Safaei, T.S., Sargent, E.H., Kelley, S.O., 2018. Analytical Methods Combinatorial Probes for High-Throughput Electrochemical Analysis of Circulating Nucleic Acids in Clinical Samples. Angew. Chem. Int. Ed. 3711–3716. https://doi.org/10.1002/anie.201800455.

del Río, J.S., Svobodova, M., Bustos, P., Conejeros, P., O'Sullivan, C.K., 2016. Electrochemical detection of Piscirickettsia salmonis genomic DNA from salmon samples using solid-phase recombinase polymerase amplification. Anal. Bioanal. Chem. 408, 8611–8620.

Fernández-Tejedor, M., Soubrier-Pedreño, M.Á., Furones, M.D., 2007. Mitigation of lethal effects of Karlodinium veneficum and K. armiger on Sparus aurata: changes in haematocrit and plasma osmolality. Dis. Aquat. Org. 77, 53–59. https://doi.org/10.3354/dao01816.

Fernandez, M., Camp, J., Marı, P., Galicia, X. De, Arousa, D., 2006. Characterization of NW Mediterranean Karlodinium Spp. (Dinophycae) strains using morphological, molecular, chemical and physiological methodologies. J. Phycol. 42, 1096–1112. https://doi.org/10.1111/j.1529-8817.2006.00270.x.

Figueroa, R.I., Garcés, E., Bravo, I., 2010. The use of flow cytometry for species identification and life-cycle studies in dinoflagellates. Deep. Res. Part II Top. Stud. Oceanogr. 57, 301–307. https://doi.org/10.1016/j.dsr2.2009.09.008.

Hocek, M., Fojta, M., 2011. Nucleobase modification as redox DNA labelling for electrochemical detection. Chem. Soc. Rev. 40, 5802–5814. https://doi.org/10.1039/c1cs15049a.

Jauset-Rubio, M., Svobodová, M., Mairal, T., McNeil, C., Keegan, N., El-Shahawi, M.S., Bashammakh, A.S., Alyoubi, A.O., O'Sullivan, C.K., 2016a. Aptamer lateral flow assays for ultrasensitive detection of β-conglutin combining recombinase polymerase amplification and tailed primers. Anal. Chem. 88, 10701–10709.

Jauset-Rubio, M., Svobodová, M., Mairal, T., McNeil, C., Keegan, N., Saeed, A., Abbas, M.N., El-Shahawi, M.S., Bashammakh, A.S., Alyoubi, A.O., O´Sullivan, C.K., 2016b. Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay. Sci. Rep. 6, 37732.

Junhui, Z., Hong, C., Ruifu, Y., 1997. DNA based biosensors. Biotechnol. Adv. 15, 43–58. https://doi.org/10.1016/S0734-9750(97)00003-7.

Kerman, K., Kobayashi, M., Tamiya, E., 2004. Recent trends in electrochemical DNA biosensor technology. Meas. Sci. Technol. 15. https://doi.org/10.1088/0957-0233/15/2/R01.

Kielkowski, P., Fanfrlík, J., Hocek, M., 2014. 7-aryl-7-deazaadenine 2′-deoxyribonucleoside triphosphates (dNTPs): better substrates for DNA polymerases than dATP in competitive incorporations. Angew. Chem. Int. Ed. 53, 7552–7555. https://doi.org/10.1002/anie.201404742.

Toldrà, A., Andree, K.B., Fernández-tejedor, M., Diogène, J., Campàs, M., 2018. Dual Quantitative PCR Assay for Identification and Enumeration of Karlodinium Veneficum and Karlodinium Armiger Combined with a Simple and Rapid DNA Extraction Method.

Wang, J., 1999. Towards genoelectronics: electrochemical biosensing of DNA hybridization. Chem. Eur J. 5, 1681–1685 https://doi.org/10.1002/(SICI)1521-3765(19990604)5:6 < 1681::AID-CHEM1681 > 3.0.CO;2-U.

Wlassoff, W. a, King, G.C., 2002. Ferrocene conjugates of dUTP for enzymatic redox labelling of DNA. Nucleic Acids Res. 30, e58 https://doi.org/e58\r10.1093/nar/gnf058.