Combination of ferrocene decorated gold nanoparticles and engineered primers for the direct reagentless determination of isothermally amplified DNA

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Abstract
In this work, we designed a reagent-less DNA sensor exploiting a combination of gold nanoparticles, modified primers, and isothermal amplification, and determining *Karlodinium armiger*, a toxic microalgae, as a model analyte to demonstrate this generic platform. Colloidal gold nanoparticles with an average diameter of 14± 0.87 nm were modified with a mixed self-assembled monolayer of thiolated 33-mer DNA probes and (6-mercaptohexyl) ferrocene. Modified primers, exploiting a C3 spacer between the primer-binding site and an engineered single stranded tail, were used in an isothermal recombinase polymerase amplification reaction to produce an amplicon with a central duplex flanked by two single stranded tails. These tails were designed to be complementary to a gold electrode tethered capture oligo probe, and an oligo probe immobilised on the gold nanoparticles, respectively. The time required for hybridisation of the target tailed DNA with the surface immobilized probe and reporter probe immobilised on AuNPs was optimised to be 10 min, in both cases. Amplification time was further optimised to be 40 min to ensure the maximum signal. Under optimal conditions, the limit of detection was found to be 1.6 fM of target dsDNA. Finally, the developed biosensor was successfully applied to the detection of genomic DNA extracted from a seawater sample that had been spiked with *K. armiger* cells. The demonstrated generic electrochemical genosensor can be exploited for the detection of any DNA sequence and ongoing work is moving towards an integrated system for use at the point-of-need.
Keywords: DNA detection; Modified primers; Functionalized gold nanoparticles; Electrochemical detection; Isothermal amplification; Recombinase polymerase amplification; *Karlodinium armiger* sequence in seawater

Declarations

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Code availability:NOT

Introduction

The electrochemical determination of specific DNA sequences has been credited as a sensitive, low-cost, and portable analytical approach with application in many diverse areas[1]. The polymerase chain reaction (PCR) is routinely used for the amplification of DNA, and despite the fact that a number of potential solutions, such as integrated microsystems, have been developed for deployment of PCR to non-laboratory settings, the inherent power and control requirement to run thermal cycling processes hinders its implementation at the point of need, particularly in resource limited settings. To address this limitation several alternative techniques for isothermal DNA amplification have been developed, including the recombinase polymerase amplification (RPA), which has several advantages over other isothermal techniques due to its simplicity, sensitivity and rapid amplification at a constant temperature between 25–42 ºC, without the need for tight temperature control [2].

In spite of the high efficiency, simplicity, and speed of RPA, the detection of RPA amplicons via hybridisation using electrochemical biosensors requires post-amplification processing, such as the generation of single-stranded DNA (ssDNA), for subsequent detection via hybridisation. Several techniques have been used to generate ssDNA from double-stranded DNA (dsDNA) amplicons, including thermal denaturation, heat/alkaline treatment of avidin/biotin-dsDNA and asymmetric PCR [3,4].

To facilitate the detection of the target sequence, we have developed a novel strategy for detecting DNA amplification products without the need to create ssDNA, by using primers modified with a carbon spacer (C3, C8, C18), which effectively blocks elongation. This results in a duplex amplicon flanked by two single-stranded DNA tails. These tails facilitate detection via direct hybridisation with surface immobilised probes designed to be complementary to one of the tails, and with a labelled reporter probe complementary to the other tail. This approach has been used in both PCR [5] and RPA [6] amplification, and even though this approach obviates the requirement for post-amplification generation of ssDNA, a labelled reporter probe for hybridisation to the other tail is still required. The assay consists of an initial hybridisation of the tailed amplicon with the immobilised probe, a subsequent washing step, followed by addition of the labelled probe, further washing, and finally detection.

The use of solid phase amplification with a biotin labelled reverse primer for the optical detection of *Yersinia pestis*, was explored as a means of avoiding the need to hybridise to a reporter labelled probe, achieving a limit of detection (LOD) of 0.3 fM [7]. This approach was also exploited for solid-phase amplification on gold electrodes for the electrochemical detection of *Piscirickettsia salmonis* and *Francisella tularensis,* achieving LODs of 5 x 10^{-8} µg/mL genomic DNA [8] and 20 fM [9], respectively. However, addition of a reporter (streptavidin-HRP) was required, and to avoid this additional step. A HRP-labelled reverse primer was used and the detection limit was lowered by one order of magnitude [10]. In an alternative approach, we recently developed an electrochemical genosensor using a HRP-labelled primer in solution phase amplification, followed by solid-phase detection [11].

Whilst this HRP label provides high sensitivity, due to the catalytic behaviour of the enzyme and its high turnover rate, enzyme labels require extra washing and substrate addition steps. Indeed, the assay protocol could be significantly simplified if an alternative to enzymes is implemented. It is, therefore, highly desirable to develop ultrasensitive electrochemical reagentless DNA biosensors [12, 13].

Electrochemical impedance spectroscopy (EIS) has been used widely in the development of reagent-less DNA sensors, due to the ability of this technique to measure the fractional change in the charge transfer resistance as a result of the hybridisation event on the electrode surface. Examples include a DNA sensor based on pyrrolidinyl peptide nucleic acid immobilised on a paper-based carbon electrode [14], as well as a genosensor based on a self-assembled nanocomposite of reduced graphene oxide and modified manganese(III)tetraphenyl-porphyrin[15]. In an another approach reported, poly(3,4-ethylene-dioxythiophene)-modified electrodes have been used, where the hybridisation event changes the electrochemical properties of the polymer, which were probed using differential pulse voltammetry[16]. Another approach for the enzyme-free detection of mRNA was developed using a combination of catalysed hairpin assembly, the hybridisation chain reaction and methylene blue as a redox probe[17]. The use of ferrocene-modified reporter probes has also been described, using the Velox™ technology, where a PCR product is hybridised with a ferrocene -labelled single-stranded DNA probe, and this hybrid is incubated with a double-strand specific exonuclease which facilitates cleavage of the terminal nucleotide, with the liberated mononucleotide-ferrocene label being detected on the surface of a screen-printed electrode using differential pulse voltammetry [18]. In an approach similar to what we propose in the work reported here, gold nanoparticle/streptavidin conjugates covered with 6-ferrocenylhexanethiol were used for the ultrasensitive detection of DNA. Detection was based on a 30-mer immobilised on a gold electrode via different SAM approaches, which hybridises with a complementary biotinylated 30-mer, followed by the addition of the AuNP-SA-Fc complex [19]. In the follow up paper by the same group, the same approach was applied to the detection of the HBV pre-S

gene extracted from a serum sample, which was amplified using PCR, and detected via hybridisation between a 27-mer immobilised probe and a 28-mer biotinylated probe, again achieving very low detection limits[20].

Nanoparticles such as gold, platinum, silver and quantum dots have also been used in the development of reagentless DNA sensors. Silver-dendrimer nanocomposites have been used as oligonucleotide labels, but these require an additional step of dissolution of the silver nanoparticles in nitric acid, which is then followed by anodic stripping voltammetry, achieving an LOD of 0.78 pM after 1-hour target incubation [18]. Quantum dot labels have also been reported but this approach again requires acid dissolution followed by stripping voltammetry of the Cd(II) ions. This gene assay displays an LOD as low as 33 aM within 3.5 hours of assay time [22]. To avoid the need for the additional step of dissolution of the label in acid, the use of gold nanoparticles (AuNPs) as a label and direct chronoamperometric detection was reported. The method enabled the determination of 0.8 Leishmania parasites per mL of blood (8×10^{-3} parasites per DNA amplification reaction) [23].

In the work reported herein, we describe an electrochemical DNA sensor, coupling tailed primers with isothermal amplification and AuNPs decorated with a mixed self-assembled monolayers (SAMs) of thiolated ferrocene and thiolated DNA probes, with the overall objective of achieving an ultrasensitive, reagentless and simple platform for the direct detection of DNA amplicons.

Materials and methods

Materials

RPA TwistAmp® basic kit was purchased from TwistDx Ltd. (Babraham, United Kingdom). DreamTaq DNA polymerase kit was purchased from Fisher Scientific (Madrid - Spain). DNA Clean & Concentrator-5™ kit was purchased from Zymo Research (Irvine, USA). 2 mm diameter gold working electrodes (CHI101), Ag/AgCl reference electrode with porous Teflon tip, and 0.3 µm and 0.05 µm α-alumina powder were obtained from CH Instruments (Austin, USA). (6-mercaptohexyl)ferrocene was purchased from Sigma–Aldrich (Barcelona, Spain) and dissolved in ethanol. Dithiol 1,6-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15-pentaoxahexa-decane) (DT1) was obtained from SensoPath Technologies (Bozeman, MT, USA). Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Scharlau (Barcelona, Spain). DNA probes, DNA primers, synthetic analogues of *Karlodinum armiger* and *Karlodinium veneficum* targets were supplied by Biomers GmbH (Ulm, Germany) as lyophilised powder, reconstituted in RNase and DNase-free water and used without further purification. Genomic DNA samples were supplied by IRTA (Cataluña, Spain).

Table 1: DNA target, probe and primers sequences used in this work

Name	**Nucleotide sequence (from 5′ to 3′)**
Karlodinum armiger target	ata gct tca cag cag agg tta caa cac caa tgc tgc tcc gct acc cgc gat ctc atg cac cag gga agc ttc aag aca ccc cta ccc ccg tgc agg agc tca caa aga aag ttc aca gtg aga tgg ttg gat gtg tgt
Thiolated capture probe	ttc att gag ttc gtc gta at ttt ttt ttt ttt ttt-SH
Tailed forward primer	att acg acg aac tca atg aa – C3 Spacer - ata gct tca cag cag agg tta caa c
Tailed reverse primer	tgt aaa acg acg gcc agt – C3 Spacer - aca cac atc caa cca tctt cac tg
Reporter probe	SH -ttt ttt ttt ttt ttt act ggc cgt cgt ttt aca
Unmodified forward primer	ata gct tca cag cag agg tta caa c
Unmodified reverse primer	aca cac atc caa cca tYt cac tg
Karlodinum veneficum target	ata gct tcg cag aca aag gtg aat ccc aat gct gct cca cta ccc gcg aac tgc taa cgc cag ggt gcg gaa gag aac tac ccc aac ccc cgc gca aga gct cac aaa gaa gtt cac agt gaa atg gtt gga tgt gtg t

PCR amplification of *Karlodinium amiger* ssDNA target to produce dsDNA and tailed templates

Single stranded *Karlodinium amiger* target was amplified using conventional PCR to dsDNA for use as a template in the RPA assay. PCR amplification was performed using DreamTaq DNA polymerase kit - with the following volumes: 10 µL 5× buffer, 5 µL dNTPs (2 mM), 5 µL $MgCl_2$ (50 mM), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 0.5 µL BSA (50 mg/mL), 27 µL H_2O, 0.5 µL of 5 U/µL polymerase and 1 µL of 5 nM ssDNA template. All PCR reactions were heated at 95 °C for 2 min, followed by 20 rounds of PCR, with a denaturing step at 95 °C for 30 s, annealing step at 58 °C for 30 s, and an elongation step at 72 °C for 30 s. A final extension step was performed at 72 °C for 5 min. PCR amplification was carried out in an iCycler Thermal Cycler (Bio-Rad Laboratories, Barcelona, Spain). The same protocol was used exploiting tailed primers instead of unmodified primers to produce tailed amplicons to be used in the optimisation experiments.

Double-stranded/tailed PCR products were analysed using agarose gel electrophoresis. Four µL of PCR products was run with 4 µL of loading buffer in a 3 % (w/v) agarose gel stained with GelRed™ nucleic acid stain and visualized with a UV lamp (λ = 254 nm).

PCR products were purified using DNA Clean & Concentrator™ kit by mixing 5 volumes of DNA binding buffer to each volume of the PCR products and the mixture transferred to the Zymo-Spin column and centrifuged for 30 sat 10000 g, after which the column was washed three times with 200 µL DNA wash buffer and additionally centrifuged for 30 s at 10000 g. Finally, 10 µL of water were added directly to the column matrix, incubated for one minute and then centrifuged for 30 sat 10000 g to elute the dsDNA. Purified dsDNA was quantified by UV spectrophotometry with a NanoDrop™ 2000 (Thermo Fisher Scientific) at λ = 260 nm.

Preparation of colloidal gold nanoparticles

AuNPs with an average diameter of 14 nm were prepared by citrate reduction of $HAuCl_4$, followed a slightly modified reported method [24]. All glassware was cleaned with aqua regia ($HCl:HNO_3$ = 3:1), rinsed with deionised water and dried before use. An aqueous solution of sodium citrate (2.0 mL, 1 wt.%) was rapidly added to a boiling $HAuCl_4$ solution (50 mL, 0.01 wt.%) under vigorous stirring conditions. The colour of the solution changed slowly from purple to red, indicating formation of the AuNPs. The solution was refluxed for an additional time of 15 min, maintaining vigorous stirring conditions. After the heat source was removed, the solution was stirred continuously until it reached room temperature. Finally, the cooled solution was stored at 4 ˚C.

Preparation of ferrocene/reporter probe-AuNP conjugation

The optimised conjugation of Ferrocene/reporter probe-AuNP was prepared by mixing 10 µL of 100 µM thiolated DNA and 10 µL of 3 mM (6-Mercaptohexyl) ferrocene with 1 mL of 3.4 nM AuNPs. The solution was left to incubate for 24 hours at room temperature. Subsequently, the conjugate was centrifuged at 6797g for 30 minutes at 22°C and the pellet was re-suspended three times in 500 µL Milli-Q water in order to clean the conjugate and to remove any free DNA or ferrocene. The conjugate was then re-suspended in 50 µL Milli-Q (final concentration = 68 nM modified AuNPs). For detection, 4 µL of modified gold nanoparticles (68 nM modified AuNPs) were drop-casted onto the electrode surface and incubated for 10 min at 37 °C to hybridise with the reporter probe.

Solution phase RPA with solid phase detection

The target (synthetic dsDNA/genomic DNA) was first amplified using isothermal recombinase polymerase amplification (RPA) utilising the aforementioned tailed forward and reverse primers that were developed previously (Table 1) [25]. Subsequently, detection was carried out by hybridisation to a capture probe immobilised on gold electrodes, followed by hybridisation with the AuNP-linked reporter probe, and finally detected electrochemically.

RPA assay

The RPA amplification mixture was prepared according to the manufacturer's instructions, by mixing 2.4 µL of 10 µM tailed reverse primer and 2.4 µL of 10 µM tailed forward primer, 2 µL dsDNA template, 11 µl DNAse free water and 29.5 µL 1× rehydration buffer. The lyophilised pellet was then added to the mixture. The reaction was finally initiated by addition of 2.5 µL of 280 mM magnesium acetate to a final volume of 50 µL, and the solution was immediately mixed by vortex. Amplification was performed for the defined time interval at 37 °C.

Electrode surface functionalization and electrochemical detection

The gold electrode surface was polished with suspensions of 0.3 µm and then 0.05 µm alumina particles followed by rinsing with Milli-Q water and ethanol and finally dried with N_2 gas. The gold surface was functionalized using a co-immobilisation approach by drop-casting 4 µL of 100 µM DT1 and 5 µM thiolated capture probe in 1 M KH_2PO_4. Formation of the SAMs was left to take place overnight at room temperature under a water-saturated atmosphere, and the electrodes were then rinsed with Milli-Q water and finally dried with N_2 gas.

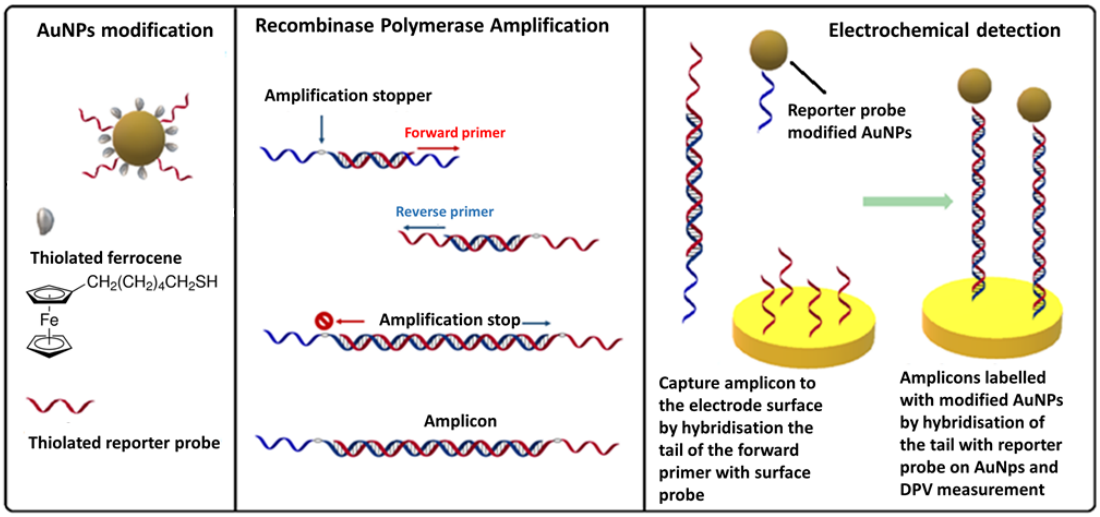

Figure 1: Schematic of gold nanoparticle modification, isothermal amplification using tailed primers and electrochemical detection.

Long-term stability studies of probe-functionalised electrodes were not carried out as we have previously carried out two sets of extensive storage stability studies with functionalised gold sputtered [21, 26] and printed circuit board electrodes [22, 27], and clearly demonstrated that the functionalised electrodes have a storage stability of >2 years when stored at either room-temperature or under refrigerated conditions, using a stabiliser (e.g. Stabilcoat), or even in the absence of a stabilizer. In addition, we did not carry out studies for regeneration and re-use of the sensors using high / low pH buffers, or hot water, as the developed generic genosensor platform is planned to be for single, and not repeated use.

Amplified PCR/RPA product (4 µL) was drop-casted onto the electrode surface and incubated for the defined time at 37 °C to hybridise with the capture probe. This incubation was carried out in a humidity-saturated chamber to avoid problems due to evaporation. Following incubation, the electrodes were sequentially washed with Tris buffered saline (pH 8) under stirring. After washing, 4 µL of modified AuNPs were drop-casted onto the electrode surface and incubated for the defined time at 37 °C to hybridise with the reporter probe. Finally, the electrodes were washed with Tris buffered saline (pH 8) under magnetic stirring.

All electrochemical measurements were performed using an Autolab PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System software (GPES) (Metrohm Autolab, The Netherlands). The three-electrode system includes 2 mm gold working electrodes, Ag/AgCl electrode as the reference and platinum wire as the counter electrode. DP voltammograms were recorded in PBS (pH 7.4) solution at a potential range from 0 to 0.7 V vs Ag/AgCl reference electrode, using a step potential of 7 mV with 50 mV/s scan rate. The DPV potential for oxidation of the 6-(Ferrocenyl)hexanethiol occurs at 0.5V vs Ag/AgCl. The charge was calculated by dividing the peak area by scan rate. Each data point represents the mean of three measurements using three different working electrodes under the same measuring conditions.

Analysis of spiked seawater sample

Extraction of genomic DNA Genomic DNA was extracted and purified from seawater samples and analysed using quantitative PCR by IRTA (Sant Carles de la Ràpita, Spain), as previously described [22]. Briefly, seawater samples were spiked with cultured *K. armiger* cells and genomic DNA was extracted using the Biomeme (BIM) isolation kit (Biomeme, Philadelphia, USA). The sample (50 mL) was centrifuged at 3700g for 25min and the pellet re-suspended in 300µL of lysis buffer (1M NaCl, 70mM Tris, 30mM EDTA, pH 8.6). This was then transferred to a 2ml cryotube containing ca. 50µg of 0.5mm diameter zirconium glass beads (Biospec, USA) and lysed using a BeadBeater-8 (BioSpec, USA). The lysed sample (250µL) was then pumped through an ion-exchange cartridge to capture all the DNA, and the ion cartridge washed with 500µL of Biomeme Protein Wash solution, then with 750µL of Biomeme Wash Buffer and finally the genomic DNA was eluted with 500µL of Biomeme Elution buffer. The extarcted DNA was kindly given to us by Dr. Mònica Campàs and Dr. Anna Toldrà.

qPCR analysis of extracted genomic DNA qPCR was performed using an ABI 7300 thermocycler (Applied Biosystems, Thermo Fisher Scientific, Spain) with previously optimised parameters [22]. Briefly, a two-step cycling protocol was carried out, with an initial denaturation step at 95ºC for 10 min, followed by 45 cycles at 95 °C for 20 s and 58 °C for 30 s. The reaction mixture contained 10µL 2 x SYBR Green dye, 2µL of primers (5µM), 2µL of DNA extracts or calibration curve standards prepared using a serial dilution of DNA target and 2µL of DNA free water.

Electrochemical detection of genomic DNA extracted from spiked seawater sample For the detection of the genomic DNA extracted from the seawater sample, the following protocol was used. The RPA amplification mixture was prepared according to the manufacturer's instructions, by mixing 2.4 µL of 10 µM tailed reverse primer and 2.4 µL of 10 µM tailed forward primer, 2 µL dsDNA template, 11 µl DNAse free water and 29.5 µL 1× rehydration buffer, and the lyophilised pellet was added to the mixture. To initiate the reaction 2.5 µL of 280 mM magnesium acetate was added to a final volume of 50 µL, and the tube containing all the reagents were vortexed. Amplification was carried out for 40 min at 37 °C. Following amplification, 4 µL of amplified RPA product were dropcast onto the electrode surface and incubated for 10 min at 37 °C to hybridize with the capture probe. Following incubation, the electrodes were washed with Tris buffered saline (pH 8) under magnetic stirring conditions. After washing, 4 µL of modified gold nanoparticles were then added to the electrode surface and incubated for 10 min at 37 °C to hybridize with the reporter probe. Finally, following an intense wash with Tris buffered saline (pH 8) under magnetic stirring conditions, differential pulse voltammetry was carried out in PBS (pH 7.4), at a potential range from 0 to 0.7 V vs Ag/AgCl reference electrode, using a step potential of 7 mV with 50 mV/s scan rate. The charge was calculated by dividing the peak area by scan rate.

Results and discussion

Characterization of gold nanoparticles

The size and the size distribution of the AuNPs was determined using TEM imaging (Figure 2) to be 14± 0.87 nm, and their concentration was determined to be (3.4 nM) by scanning from 800 nm to 200 nm and monitoring the peak at 520 nm (Cary 100 Bio UV-visible spectrophotometer, Agilent). The concentration of the AuNPs was determined using the Beer-Lambert law, via the absorbance of the AuNPs at 520 nm and using an extinction coefficient of 3.67 x 10^{-8} $M^{-1}cm^{-1}$, as previously reported [25].

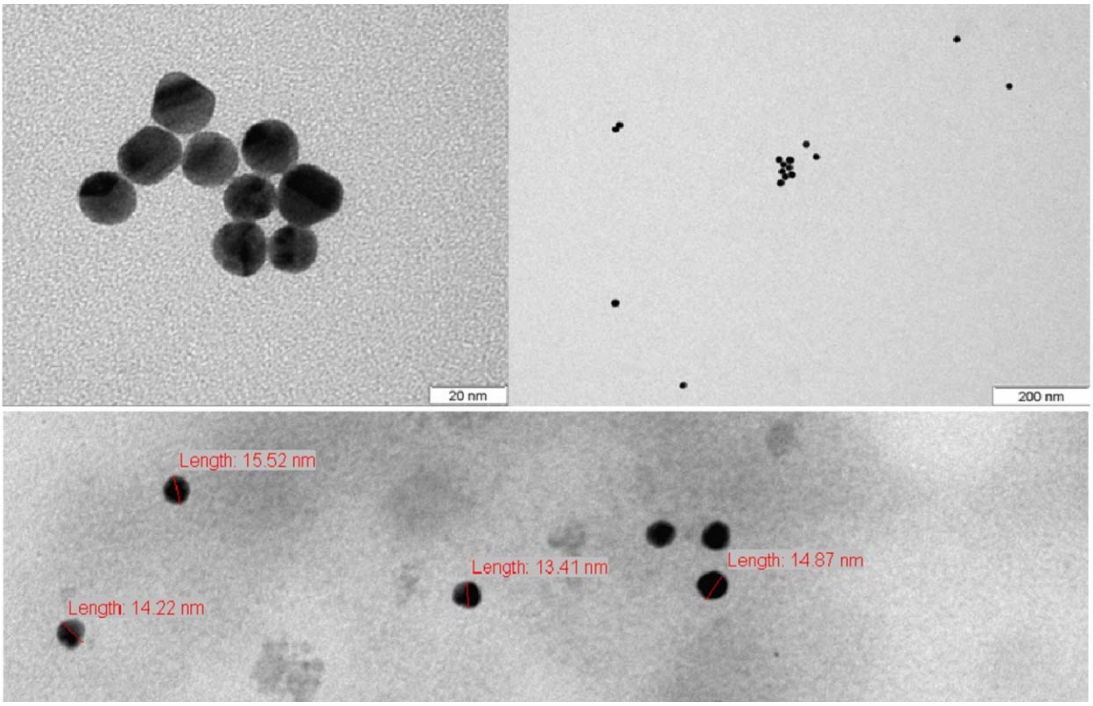

Figure 2: TEM images of gold nanoparticles

Ferrocene/reporter probe-AuNP conjugation optimisation

The AuNPs were modified via chemisorption of different ratios of thiolated ferrocene (dissolved in ethanol) and thiolated reporter probe, as outlined in Table 2.

Table 2: Ferrocene/reporter probe-AuNP conjugation conditions

	Ferrocene (3 mM)	Reporter probe (100 µM)	Gold nanoparticles (3.4 nM)
1	10 µL	1.25 µL	1 mL
2	10 µL	2.5 µL	1 mL
3	10 µL	5 µL	1 mL
4	10 µL	10 µL	1 mL
5	10 µL	20 µL	1 mL

Initially, conventional PCR was performed (using the protocol described above) using tailed primers to produce amplification products with a duplex flanked by two single-stranded DNA tails. Following purification, 4 µL of the prepared tailed dsDNA (1 nM) was drop-casted on the electrode surface and incubated to allow hybridisation with the surface capture probe for 20 min, and then with 4 µL of 68 nM modified AuNPs for a further 20 min at 37°C. The electrochemical signals obtained for each of the ferrocene:DNA ratios outlined in Table 2, (1, 2, 3, 4, 5), and the control is the electrochemical signal obtained where the tailed PCR amplicon is replaced by Tris-HCl buffer (pH 7.4), using the conditions for conjugation #4 as a label, can be seen in Figure 3B. The electrochemical signal increases with increasing reporter probe concentration, reaching a plateau at the conjugation #4. For this reason, the conjugation #4 was used in all further experiments.

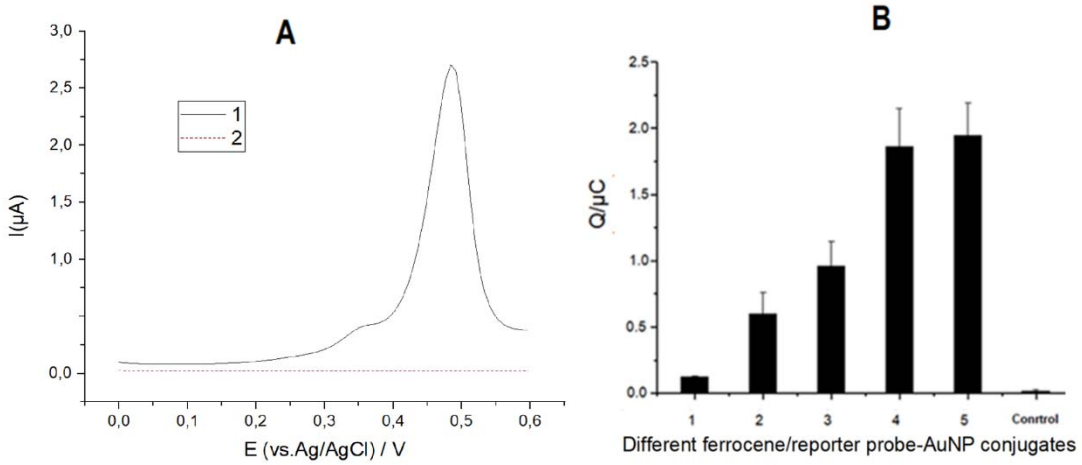

Figure 3: (A) Differential pulse voltammograms registered in the presence of tailed PCR amplicon using 1 nM template *Karlodinium armiger* (1), and a Tris-HCl control (2) using ferrocene/reporter probe-AuNP conjugation #4 as label. (B) Electrochemical evaluation of the ferrocene/reporter probe-AuNP conjugations using conditions as outlined in Table 2, and the control is just Tris-HCl (pH 7.4) in the absence of PCR amplicon. Error bars represent the standard deviation of three measurements.

Optimisation of the experimental conditions

One nM of the amplified and purified tailed target DNA in 50 mM Tris buffered saline (pH 8) containing 1 M NaCl was used to optimise the time needed for the hybridisation of the tailed amplicons with surface immobilised capture probe. The tailed DNA (4 μL) was directly drop casted onto the electrode surface and allowed to hybridise from 2–20 min at 37 °C, whilst maintaining the hybridisation time with the reporter probe constant at 20 min (Figure 4A). The tailed amplicon was replaced with Tris-HCl (pH 7.4), which acted as a control to monitor any non-specific adsorption of the functionalised AuNPs on the electrode surface. Subsequently, the time needed for the hybridisation of the surface-captured target with the reporter probe was optimised using the same strategy by varying the hybridisation time of the target with the reporter probe from 2 to 20 min, whilst maintaining the hybridisation time with the surface probe constant at 20 min (Figure 4B). It can be clearly seen that 10 min was sufficient for each hybridisation step.

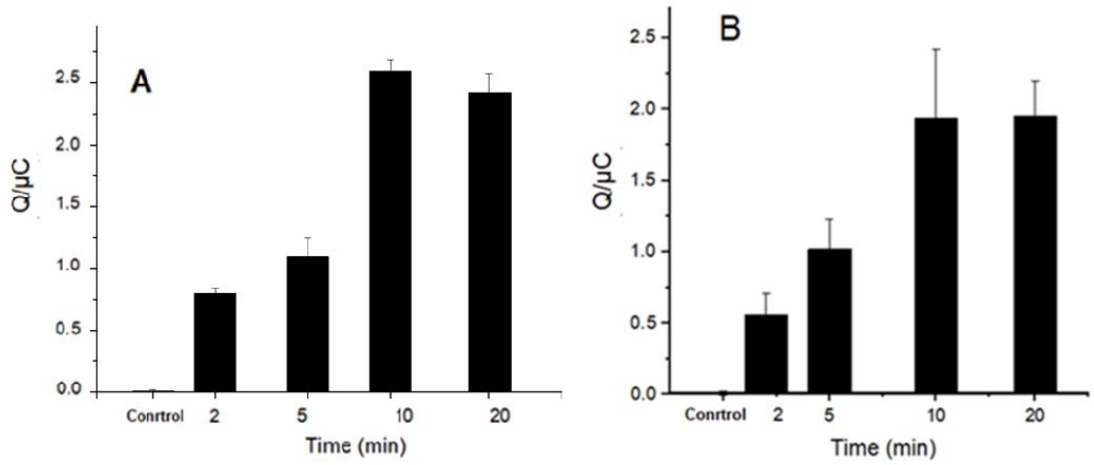

Figure 4: (A) Effect of duration of hybridisation time of 1 nM tailed target with capture probe surface tethered on gold electrode. (B) Effect of duration of hybridisation time of 1 nM tailed target with the functionalized AuNPs. Experimental conditions as described in the text. The control represents replacement of the tailed amplicon with Tris-HCl. Error bars represent the standard deviation of three measurements.

Optimisation of amplification time

The optimisation of the amplification time was carried out by amplifying 2 pM dsDNA template for 10, 20, 30, 40 and 50 min, using the protocol described above. Tailed primers were used to perform the liquid phase amplification at 37 ºC using a master mix of 100 µL. Subsequently, 15 µL aliquots were taken from the master RPA reaction after each of 10, 20, 30, 40, and 50 min and the RPA reaction rapidly stopped by immediate freezing. For the no template control (NTC), DNA-free water was subjected to the same amplification conditions for 40 min. Following RPA, 4 µL of the amplification products were drop-casted onto the electrode surface and left to hybridise with the surface immobilized capture probe for 10 min, followed by hybridisation with the AuNP-Fc labelled reporter probe. DPV measurements were conducted using the conditions described above. As can be seen in Figure 5, increasing amplification times resulted in increasing levels of amplicons produced, reaching a plateau at ca. 40 min, after which further amplification did not improve the amplification yield.

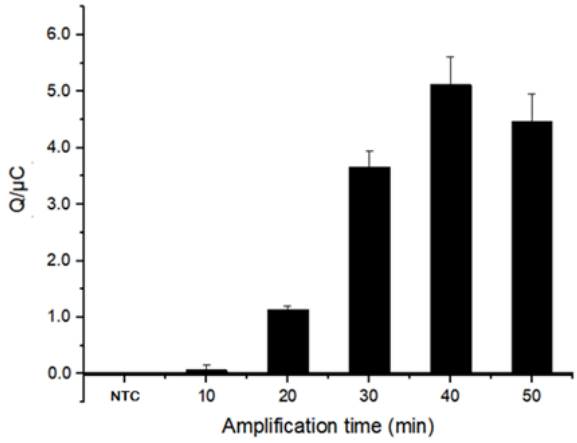

Figure 5: Electrochemical response of RPA amplicons following 10, 20, 30, 40 and 50 min amplification. NTC indicates the signal obtained following amplification of DNA-free water for 40 min. 2 pM target was used as template, 10 min hybridisation with capture probe and 10 min hybridisation with reporter probe. Error bars represent the standard deviation of three measurements.

Using optimised conditions, a calibration plot was obtained using 8-fold serial dilutions of the starting dsDNA template from 20 pM to 20 aM. The LOD was found to be 1.6 fM and corresponds to the concentration of the analyte at the mean blank signal plus three times the standard deviation of the blank.

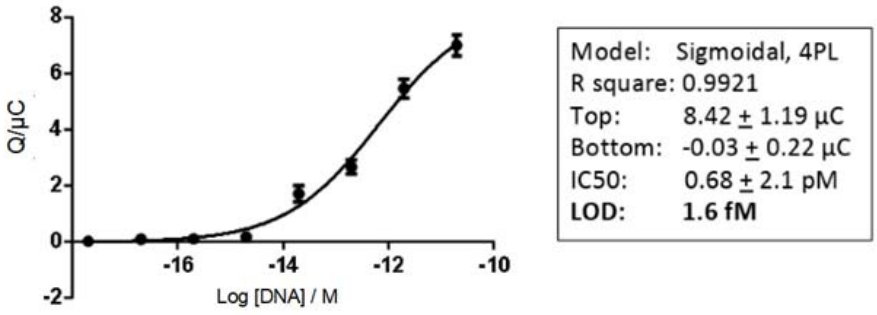

Figure 6: Calibration plot obtained by amplifying a range of concentrations (20 pM to 20 aM) of dsDNA template starting concentrations after 40 min RPA and 10 min hybridisation with capture probe and 10 min hybridisation with reporter probe.

Table 3 gives an overview of the current detection limits that can be achieved with selected electrochemical sensors which contain nanomaterials as an electrochemical label. this table shows that our sensor has the shortest assay time, at the same time it doesn't require post-amplification sample treatment.

Table 3: Comparison of the detection time and LOD of selected nanomaterials-based electrochemical genosensors.

Electrode	ssDNA generation	Nanostructured material used	Detection Time	Detection method	LOD	Ref.
GCE	Required	AuNPs	2h	DPV	350 aM	[26]
GCE	Required	dsDNA templated CuNPs	2h	DPV	30 aM	[19]
SPCE	Required	Capture probe modified AuNPs	1h	DPV	214 pM	[27]
Gold	Required	CNTs and AuNPs	1h	LSV	5.2 fM	[28]
Gold	Required	DNA-templated silver nanoparticles (AgNPs)	1h	DPV	4.7 aM	[29]
Gold	Not Required	Ferrocene decorated gold nanoparticles	20 min	DPV	1.6 fM	This work

The specificity of this sensor towards *Karlodinium armiger* target was evaluated by exposure to 2 pM *Karlodinium veneficum* target that had been amplified for 40 min. As expected, only the *Karlodinium armiger* gave a positive electrochemical signal (Fig 7A), highlighting the specificity of the system.

Genomic DNA was extracted and purified from seawater samples spiked with *K. armiger* cells. The extracted DNA was analysed using quantitative PCR by IRTA (Sant Carles de la Ràpita, Spain), employing extraction and purification procedures previously reported [22, 24]. A sample containing *Karlodinium armiger* target sequence (Ge +ve) and a control sample not containing the *Karlodinium armiger* sequence (Ge -ve) were analysed, and as can be seen in Figure 7B a significant positive electrochemical signal was observed in the case of the positive sample, whilst a markedly lower response was observed for the seawater sample not containing the *Karlodinium armiger* target.

The concentration of the genomic DNA has been calculated using the regression equation of the calibration plot (Fig 6), and it found to be 3 fM, which is in excellent correlation with the results obtained from qPCR for the same sample (3.4 fM). These data justify the suitability of the developed method for the determination of *Karlodinium armiger* target sequence (Ge +ve) in real samples at the fM concentration level, and demonstrates the wide potential applicability of the generic approach to other DNA sequences.

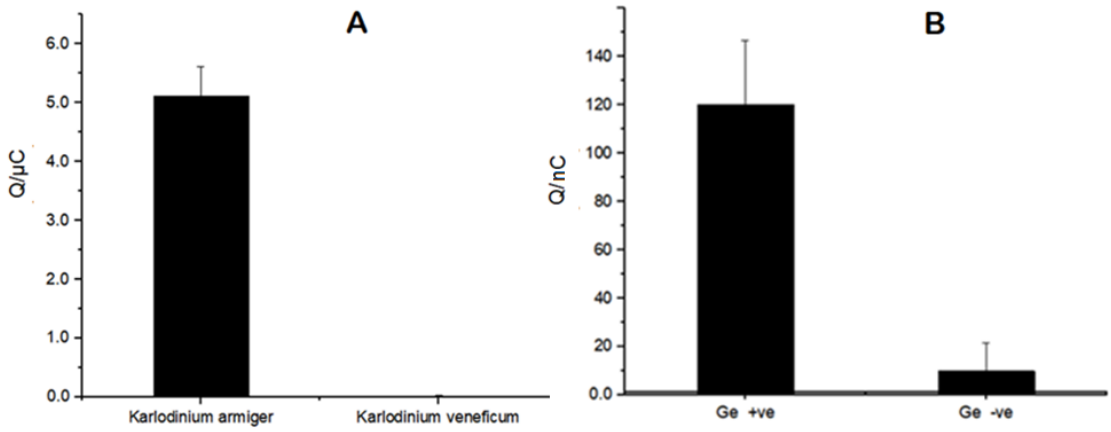

Figure 7: (A) Specificity of developed genosensor. (B) Application of genosensor to DNA extracted from real seawater sample containing K. armiger (Ge +ve) and not containing K. armiger (Ge –ve). Measurement conditions are described in text.

Conclusions

In this work, the use of AuNPs modified with thiolated ssDNA and (6-mercaptohexyl)ferrocene for the reagentless electrochemical sensing of isothermally amplified amplicons of a model DNA target was demonstrated. The AuNP-facilitated labelling with multiple ferrocene molecules, thus contributing to significant signal enhancement without increase of non-specific signal. Modified primers were used to produce an amplicon with a central duplex flanked by two single stranded tails. These tails were designed to be complementary to a gold electrode tethered capture oligo probe as well as an oligo probe immobilised on the AuNPs. The time required for hybridisation of the target DNA with the surface immobilised probe and reporter probe was optimised to be 10 min, in both cases. Using the optimum conditions of amplification and hybridisation, the LOD obtained was 1.6 fM for synthetic dsDNA target. At the same time, the selectivity of the sensor was demonstrated using *Karlodinium veneficum* DNA controls. Furthermore, the developed genosensor was utilised for the amplification and detection of genomic *Karlodinium armiger* extracted from a seawater sample. Future work will focus on the direct labelling of one of the primers with the ferrocene-decorated AuNPs and incorporation of the assay into a lateral flow format, thus avoiding any wash steps or requirement for multiple hybridisations.

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Graphical Abstract