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Solid-phase recombinase polymerase amplification using ferrocene-labelled dNTPs for electrochemical detection of single nucleotide polymorphisms

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ABSTRACT

Hypertrophic cardiomyopathies (HCM) are the principal cause of sudden cardiac death in young athletes and it is estimated that 1 in 500 people have HCM. The aim of this work was to develop an electrochemical platform for the detection of HCM-associated SNP in the Myosin Heavy Chain 7 (MYH7) gene, in fingerprick blood samples. The platform exploits isothermal solid-phase primer elongation using recombinase polymerase amplification with either individual or a combination of four ferrocene-labelled nucleoside triphosphates. Four thiolated reverse primers containing a variable base at their 3' end were immobilised on individual gold electrodes of an array. Following hybridisation with target DNA, solid phase recombinase polymerase amplification was carried out and primer elongation incorporating the ferrocene labelled oligonucleotides was only detected at one of the electrodes, thus facilitating identification of the SNP under interrogation. The assay was applied to the direct detection of the SNP in fingerprick blood samples from eight different individuals, with the results obtained corroborating with next generation sequencing. The ability to be able to robustly identify the SNP using a 10 μL fingerprick sample, demonstrates that SNP discrimination is achieved using low femtomolar (ca. 8×10^5 copies DNA) levels of DNA.

1. Introduction

Single nucleotide polymorphisms (SNPs) are the most frequent sequence variations in humans (Marshall, 1997). Array-based detection technologies enable the simultaneous interrogation of several SNPs on a single platform using only few microliters of sample, are based either on detection of hybridisation or single base extension, and typically use fluorescence transduction.

Single base extension (SBE) of primers (Sokolov, 1990), which exploits the principle of the Sanger sequencing method (Sanger et al., 1977) using dideoxynucleotides (ddNTP), has been applied to the detection of genetic diseases (Kuppuswamy et al., 1991) and point mutations (Picketts et al., 1992). Solid-phase SBE was subsequently developed and also applied to the detection of single nucleotide mutations (Syvänen et al., 1990, 1993) and polymorphisms using array-based Primer Extension, commonly referred to as APEX (Pastinen et al., 1996; Shumaker et al., 1996). In APEX, primers designed to hybridise to single stranded DNA (generated from PCR products) one single base downstream of a SNP site, are surface-tethered on a microarray chip via their 5′ termini. Following hybridisation, fluorescently labelled 2′,3′-dideoxyribonucleoside triphosphates (ddNTPs) are added and after SBE, the SNP is identified via the fluorescent label incorporated (Pastinen et al., 1997).

Electrochemical detection of SBE using redox-labelled ddNTPs has been reported as an alternative to fluorescence (Brazill and Kuhr, 2002; Brazill et al., 2003; Hebert and Brazill, 2003) and we recently reported the preparation and incorporation of ddNTPs labelled with diverse redox labels and their application to SNP detection using solid-phase single base extension (Chahin et al., 2018; Debela et al., 2016). However, redox-labelled ddNTPs are expensive and quite complex to produce and

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the use of redox-labelled dNTPs in solid-phase primer elongation is an attractive alternative. In this approach, primers containing a variable base (A, G, T, C) at their 3′ end are immobilised via their 5' ends. Single stranded DNA (ssDNA) containing the SNP to be interrogated is generated from PCR amplicons and allowed to hybridise to all four immobilised probes. Solid-phase amplification is subsequently carried out using *Taq* polymerase and dNTPs containing Cy3-dUTP. Primer elongation and fluorescence signal is only observed where there is full complementarity between the immobilised primer and ssDNA (Erdogan et al., 2001; Huber et al., 2001; Trau et al., 2002). Whilst this approach is elegant and compatible with existing array platforms for multiplexed detection, it requires previous nucleic acid amplification and generation of ssDNA. To address this, we wanted to explore the possibility of combining solid-phase amplification and primer elongation. Whilst solid-phase PCR, where one primer is surface-immobilised and the other primer is in solution, has been widely reported (Chin et al., 2017; Damin et al., 2016; Gu et al., 2019; Hoffmann et al., 2012; Hung et al., 2017), surface chemistries typically used for the immobilisation of thiolated DNA probes on gold electrodes are not compatible with the elevated temperatures required (Civit et al., 2010) and we thus explored the possibility of isothermal amplification.

Various methods have been developed for isothermal amplification including the Helicase Displacement Assay (HDA), Strand Displacement Assay (SDA) Recombinase Polymerase Amplification (RPA) and Loop-mediated Isothermal Amplification (LAMP), amongst others (Bodulev and Sakharov, 2020). Whereas the most commonly reported technique to date is LAMP, RPA is particularly attractive as it requires just two primers per target, can be carried out at low temperatures (22–45 °C), does not require tight temperature control and is rapid, making it highly compatible with point-of-care applications (Magriña and O'Sullivan, 2018).

RPA was developed by Piepenburg and coworkers (Piepenburg et al., 2006; Piepenburg et al., 2010) and takes advantage of proteins involved in cellular DNA synthesis, recombination and repair. RPA is initiated when a recombinase protein such as uvsX from T4-like bacteriophages binds to primers in the presence of ATP and a crowding agent (e.g. polyethyleneglycol), forming a recombinase-primer complex. This complex seeks a homologous sequence in double stranded DNA and promotes strand invasion by the primer at the cognate site, with the displaced DNA strand being stabilised by single-stranded binding proteins. The recombinase finally disassociates, and a strand displacing DNA polymerase binds to the 3' end of the primer, elongating it in the presence of dNTPs, with cyclic repetition of this process resulting in exponential amplification.

We have previously explored various formats for solid-phase RPA, including the use of biotinylated reverse primers and thiolated forward primers immobilised on microtitre plates for the detection of *Yersinia pestis* (Mayboroda et al., 2016), and on gold electrodes for the electrochemical detection of *Francisella tularensis* (del Río et al., 2014) and *Piscirickettsia salmonis* (Del Río et al., 2016). Grammoustianou et al. reported an interesting approach for the quartz-crystal microbalance based detection of solid-phase RPA, where they used cholesterol labelled reverse primer and liposomes to amplify the signal (Grammoustianou et al., 2017). Sánchez-Salcedo et al. reported the successful amperometric detection of *Salmonella* genomic DNA based on solid phase RPA to generate a 6-FAM-tagged amplicon that was detected with an anti-FITC Fab fragment conjugated to HRP (Sánchez-Salcedo et al., 2019), whilst Ichzan et al. recently reported on the electrochemical detection of solid-phase RPA of a target gene of the hepatitis B virus on ITO electrodes, where they added a small amount of forward primer to the solution phase, improving the amplification efficiency, achieving a detection limit of 0.1 fM (Ichzan et al., 2021). However, in these approaches the addition of reporters/substrates are required, and to avoid this further step, we used HRP-labelled reverse primers (Mayboroda et al., 2016) but this still required addition of enzyme substrate. Alternatively, we explored the use of gold nanoparticles co-functionalised with thiolated ferrocene and reverse primer but a further step of labelled primer addition was still needed (Al-Madhagi et al., 2021). Recently we reported on the electrochemical analysis of redox-labelled nucleotides for the orthogonal multipotential coding of all four bases (Kodr et al., 2021), and the use of redox-labelled nucleotides would facilitate direct electrochemical detection of the DNA, avoiding any further steps of substrate/reporter addition.

Based on previous work where 7-(ferrocenylethynyl)-7-deaza-2′-deoxyadenosine triphosphate and three other ferrocene modified nucleotides were observed to be enzymatically incorporated in short synthetic sequences (Brázdilová et al., 2007; Ménová et al., 2013) and longer DNA templates using PCR (Simonova et al., 2020), we explored the use of ferrocene labelled oligonucleotides for the detection of DNA. Ferrocene modified adenosine triphosphate were used in combination with natural dNTPs for the PCR based amplification of DNA from *Bacillus anthracis* CAP and PAG (Magriñá et al., 2019a) and genomic *Karlodinium armiger* DNA in real seawater samples (Magriñá et al., 2019b). Whilst these studies demonstrated the successful incorporation of ferrocene-labelled dNTPs into a PCR amplicon and subsequent detection of the labelled amplicon, the possibility of RPA-mediated incorporation of these labelled dNTPs has not yet been investigated. Whilst the use of labelled primers in RPA has been widely reported (Li et al., 2018), there are only a few recent reports of the use of labelled dNTPs, such as that of the use of Cy5-dUTPs for the detection of antibiotic resistance genes (Warmt et al., 2021), tyrosine labelled dUTPs (Suprun et al., 2021), as well as HRP-labelled dTTPs for the detection of oxacillin resistance (Butterworth et al., 2021).

There are also some recent reports of the use of RPA for the detection of single nucleotide polymorphisms (SNPs), including the use of probe-directed RPA for the detection of A and C nucleotides at the A1289C polymorphism associated with congenital heart disease (Duan et al., 2018). Lázaro et al. described allele-specific ligation combined with RPA for the detection of variants related to cardiovascular diseases (Lázaro et al., 2019), and finally allele-specific RPA for the detection of SNPs associated with multi-drug resistant tuberculosis (Singpanomchai et al., 2021).

Furthermore, unlike PCR, where genomic DNA should be extracted and purified prior to the amplification, RPA can work in the presence of several of the PCR inhibitors of such as SDS, ethanol, the anticoagulant heparin as well as hemoglobin, serum and even in whole blood (Eid and Santiago, 2016; Kersting, 2014), simplifying the pre-amplification treatment of the sample, and potentially facilitating the direct use of RPA in fingerprick blood samples.

In the work reported here, we developed a generic platform for the electrochemical detection of single nucleotide polymorphisms, based on solid-phase primer elongation on gold electrodes using ferrocene labelled oligonucleotides. As a model system for a demonstration of the proof-of-concept, we detected a SNP located in the 14q12 locus of the β-Myosin heavy chain (MYH7) gene (Maron et al., 2000; Oldfors, 2007). This SNP is a single nucleotide variation (SNV) where the reference allele contains the T base, while the presence of a G base at that position is related to cardiomyopathy. SNPs associated with cardiomyopathies have been detected using direct sequencing, single-strand conformation analysis (SSCA), denaturing gradient gel electrophoresis (DGGE), and denaturing high-performance liquid chromatography (dHPLC), all of which are expensive and time-consuming (Fokstuen et al., 2008). The developed platform exploits the use of identical reverse primers containing a variable base (A, G, T, C) at their 3′ end and immobilised through their thiolated 5' ends via chemisorption onto individual gold electrodes of an array. Initially, double stranded (dsDNA) was prepared from a synthetic ssDNA to mimic the genomic DNA and was used to optimise the ratio of ferrocene labelled:natural oligonucleotides, concentrations of the solution-phase forward primer and magnesium acetate and the duration of solid-phase amplification. Using these optimised conditions, the platform was demonstrated to effectively discriminate the electrode where primer elongation took place, thus facilitating the

detection of the allele at the SNP site. The approach was then applied to the analysis of a fingerprick blood samples, where following thermal lysis, solid-phase isothermal primer elongation was directly carried out in the lysed samples to identify the SNP, which was correlated using next generation sequencing.

2. Materials and methods

2.1. Reagents

The reagents (analytical grade) were used without further purification. Ultrapure water (18 MΩ cm) purified by a Simplicity Water Purification System (Millipore, France) was used for preparing the electrochemical solutions while the enzymatic solutions were prepared using DNase free water from Thermo Fisher Scientifics, Spain. Sodium chloride (NaCl), strontium nitrate ($Sr(NO_3)_2$), potassium hydroxide (KOH) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Thermo Fisher Scientifics (Spain), while sulphuric acid (95–97%) was received from Scharlau, Barcelona, Spain. Hydrochloric acid (35% v/v) was purchased from Panreac, and disodium Na_2EDTA, glycine-HCl and mercaptohexanol (MCH) were provided by Merck (Sigma Aldrich, Spain).

Recombinase Polymerase Amplification (RPA) kit (TwistAmp® Liquid Basic kit TALQBAS01) was purchased from TwistDX, UK and Dream Taq polymerase, GeneRuler Low Range DNA Ladder and agarose electrophoresis gel was received from ThermoFisher Scientific (Spain). Finally, DNA Clean & Concentrator kit was purchased from Ecogen (Spain).

$dA^{Fc}TP$ and $dU^{Fc}TP$ (Brázdilová et al., 2007), $dG^{Fc}TP$ (Ménová et al., 2013) and $dC^{Fc}TP$ (Simonova et al., 2020) were synthesized as previously reported, with final purities of >90%. The $dA^{Fc}TP$ and $dC^{Fc}TP$ are now commercially available from Jena Bioscience (www.bioscience.com). The HPLC purified oligonucleotides were purchased from Biomers.net, Germany and used as received. These labelled $dN^{Fc}TPs$ are stable for at least 12 months if stored at -20 °C (Gerstein, 2001). We prepared aliquots of each of the labelled $dN^{Fc}TPs$ and observed each aliquot to be stable even after freeze-thaw cycles.

The sequences used (from 5′ to 3') are listed in Table S18. Double stranded DNA to be used as a template for RPA was generated from the single stranded DNA target using PCR, as described in the SM.

2.2. Agarose gel electrophoresis

4% agarose gel stained with GelRed nucleic acid stain (Biotium) was prepared in 1X Tris-Borate-EDTA buffer (TBE) at pH 8. Five microlitres of each DNA sample were then mixed with 4 μL of DNA Gel Loading Dye (6X), loaded on the 4% agarose gel and then run in the same buffer at 100 mV for 30 min. For a better comparison between gels, the intensity of each band was obtained using Image J software and normalised using the value obtained from the ladder intensity band as the reference for each gel.

2.3. Next generation sequencing

The genomic DNA template extracted after thermal lysis was amplified using the same PCR conditions as described in SM. Modified primers, containing barcode sequences, were needed for the amplification. Different barcodes were added to the Forward primer sequences to differentiate each sample, whilst the same Reverse primer was used for all samples (Table S1). The resulting amplicon was column-purified using DNA Clean and Concentrator kit (Ecogen, Spain) (Fig. S15 and subsequently, sequenced using Ion Torrent Next-Generation Sequencing in the Centre for Omic Sciences, Eurecat Technology Centre (Reus, Spain).

FastaQ raw data was imported into the Galaxy web server for analysis (https://usegalaxy.org). The format of the data was then converted to FASTA using the "FASTAQ to FASTA converter" tool. The length of the sequences was filtered to the expected length of the amplified product (80 bp) using the option of "Filter sequences by length" in order to remove amplification and sequencing artefacts from the data sets. Unique sequences were then identified using the "Collapse" tool. The most representative sequence was chosen as the correct sequence for SNP detection.

2.4. Electrochemical measurements

The electrode arrays, microfluidics and double sided-adhesive tapes were manufactured at Fraunhofer ICT-IMM, Germany (Fragoso et al., 2011). The microfluidics manufacturing was based on a high precision milling of polycarbonate sheets and the 50 μm double-sided medical grade adhesive foil was cut by laser. The electrode arrays, fabricated using a photolithographic process, were composed of 16 individual 1 mm^2 square 150 nm-gold working electrodes having a common gold counter electrode and silver reference electrode (0.2 × 1 mm^2). The electrode array was housed inside the microfluidics via a 50 μm double-sided medical grade adhesive foil to create microchannel structures of 1 mm width. (Fig. S2). As observed in Fig. S2B, these microfluidic structures were composed of four individual 15 μL channels with an input and output to perform 4 individual reactions at the same time. Each channel contains four electrodes for each of the four primers with a different base at the 3′ end for SNP detection. Finally, the array was connected via pogo-pin connectors to an external multichannel potentiostat for electrochemical detection. The electrochemical measurements were carried out using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System (GPES) software (Eco Chemie B.V., The Netherlands).

The electrode arrays were cleaned by sonication in acetone, isopropanol, and water for 5 min each to remove the protected lacquer and were then subjected to an electrochemical cleaning. Firstly, the array was immersed in 0.1 M KOH and the potential was cycled 10 times from 0 to -1.2 V vs Ag, washed with water and subsequently cleaned in 0.1 M H_2SO_4 by cycling the potential from 0 to 1.5 V vs Ag. Finally, the electrode array was washed with water and dried with nitrogen.

Electrochemical impedance spectroscopy (EIS) was performed in 1 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M KCl in the frequency range from 100 kHz to 0.1 Hz at 0.20 V and amplitude of 5 mV. The Nyquist plots obtained were fitted to an equivalent Randles circuit to extract the value of charge-transfer resistance (R_{ct}) after each step under study.

2.5. Solid phase recombinase polymerase amplification

One microlitre of each of the four thiolated Reverse primers mixed with MCH in a 1:10 M ratio, (5 μM HS-RV-Primer and 50 μM MCH), in 1 M KH_2PO_4 solution were dispensed on individual electrodes of the array and left to self-assemble in a humidity chamber for 4 h at 37 °C. Finally, the electrodes were washed in 1 M KH_2PO_4 under stirring conditions for 5 min and dried with nitrogen.

The electrode array was then placed within a microfluidic housing composed of individually addressable four channels. The electrodes in three of the channels were used to carry out the measurements in triplicate, whilst the electrodes in the fourth one were used to evaluate the non-specific interactions of the reaction mixture with the electrode surface and/or microfluidic housing.

Subsequently a mixture containing 200 μM of total dNTPs (containing $dN^{Fc}TPs$ and natural dNTPs), RPA reagents, 0.5 μM Fw-Primer and 100 pM of PCR generated amplicon target (for optimisation experiments) or whole blood (for final assay) were injected into the channel. Maintaining the reaction temperature at 37 °C, the optimisation of different parameters (reaction time, forward primer concentration, $dN^{Fc}TPs$/dNTPs ratio and $Mg(OAc)_2$ concentration) were carried out to achieve the highest specificity of the detection (Further details in SM). Glycine-HCl, (0.1 M, pH 3) was then added to each channel and left

for 10 min before washing twice with the same solution. Finally, the channels were filled with a solution of 0.1 M glycine-HCl + 0.1 M Sr $(NO_3)_2$ (pH 3) and the electrochemical measurements carried out using SWV. SWVs were recorded from 0 to 0.6 V vs Ag using a pulse amplitude of 0.1 V, a step potential of 10 mV and a frequency of 25 Hz.

3. Results and discussion

3.1. SNP detection strategy

Fig. 1A and Fig. S1 depict the strategy employed for the detection of SNPs using solid-phase isothermal recombinase polymerase amplification (SP-RPA) for surface-tethered primer elongation using ferrocene labelled dNTPs. A set of four identical 5′-thiolated 22-mer primers containing a variable base at the 3'end, corresponding to the SNP site, was designed and thus only one of the four primers was fully complementary to the DNA target. Assay conditions were optimised so that SP-RPA, resulting in primer elongation with redox-labelled dNTPs only occurred from the fully-complementary primer-dsDNA complex, resulting in electrochemical signal being observed at one electrode and negligible signal at the other three. The RPA reagent mix was enriched with either a mixture of all four or with one of the individual dNTPs labelled with ferrocene, namely $dA^{Fc}TP$, $dU^{Fc}TP$, $dC^{Fc}TP$ and $dG^{Fc}TP$ (Fig. 1B). The ferrocene unit was used as an electroactive marker to follow the amplification reaction by recording the corresponding square wave voltammograms (SWV).

3.2. Compatibility of the RPA master mix with the ferrocene labelled dNTPs in solution-phase

The RPA master mix is a complex system and its compatibility with deoxynucleotides labelled with a bulky organometallic compound such as ferrocene was first evaluated using solution-phase amplification. Each of the four natural dNTPs were doped with increasing percentages of each of the $dN^{Fc}TP$ ranging from 10 to 100%, with 100% of the other three natural dNTPs. Individual RPA reactions were then carried out in solution using 5′-end thiolated primer fully complementary with the target dsDNA and the corresponding unmodified forward primer. For these optimisation experiments, the dsDNA target was previously produced by PCR from a synthetic amplicon. After 30 min, the reaction was stopped by inactivating the RPA enzymes by heating to 60 °C. The yield of each reaction was analysed using gel electrophoresis and compared with the reaction carried out in the absence of ferrocene labelled dNTPs. The double stranded thiolated RPA products were then immobilised on

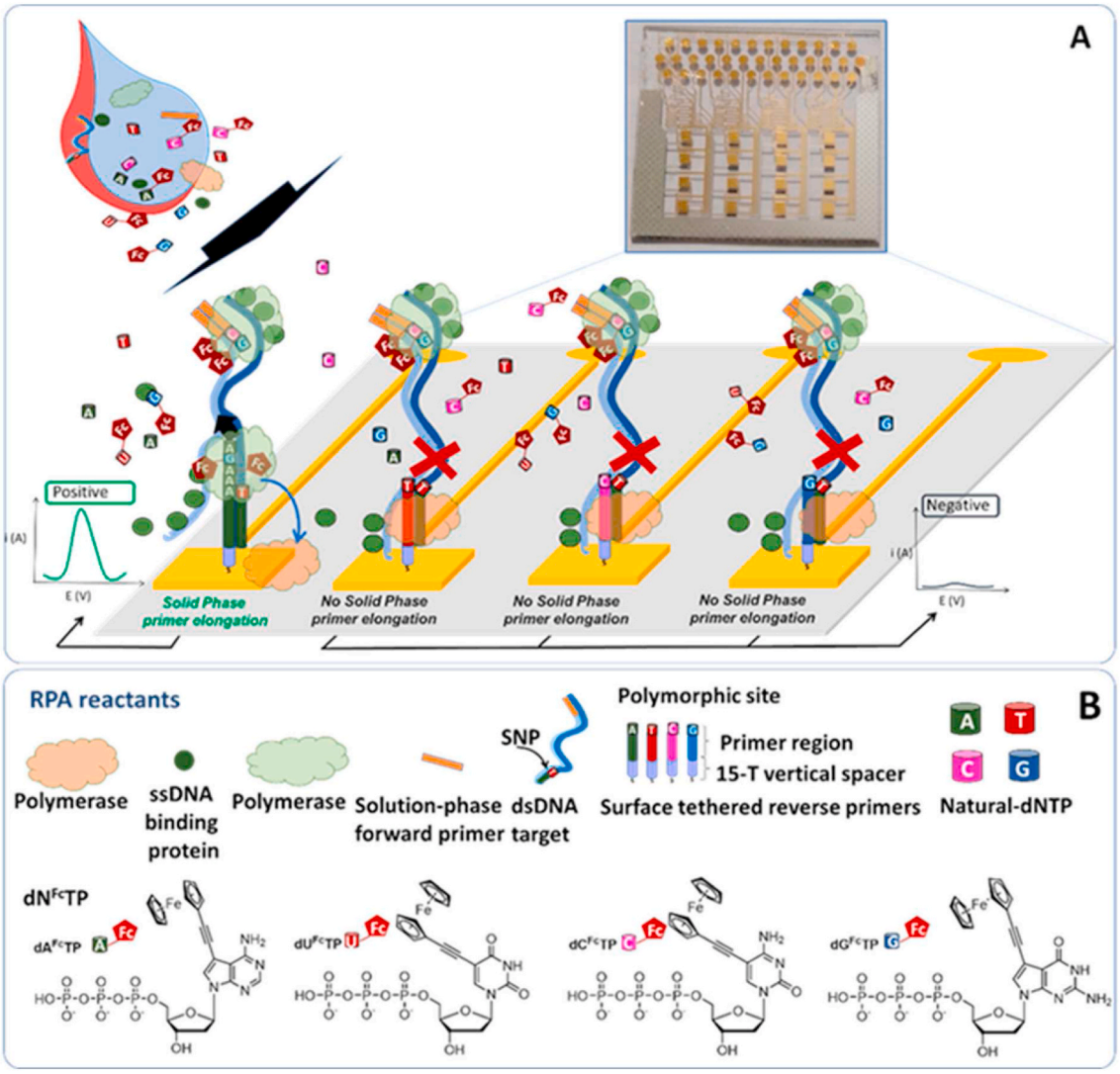

Fig. 1. (A) Schematic representation of the solid phase-RPA reaction carried out for SNP detection (Photograph of the actual electrode array in the inset). A detailed flow-through of the assay is shown in SM (Fig. S1) while magnified and detailed photographs of electrode array and microfluidics are shown in SM (Fig. S2); (B) RPA reagents and structure of ferrocene labelled dNTPs ($dN^{Fc}TP$) used for the reaction.

gold electrode surfaces via chemisorption.

In order to eliminate any dN^{Fc}TP non-specifically intercalated into the surface-tethered DNA, the duplex was denatured at acidic pH. Square wave voltammograms were recorded before and after denaturation and as can be seen in Fig. S3, this step facilitated an increased flexibility of the resulting surface-tethered ssDNA as compared to the rigid dsDNA duplex, allowing increased proximity to the electrode surface, resulting in an enhanced electron transfer and higher peak currents.

Fig. S4 shows the gel electrophoresis analysis of solution phase RPA products showing the band intensity obtained for each of the dN^{Fc}TP ratios studied and is overlayed with the corresponding oxidation peak currents measured. In general, and as expected, the efficiency of the reaction decreased with increasing percentages of modified dNTP as revealed by the gel electrophoresis. Nevertheless, the lower amount of RPA product was compensated by the higher number of ferrocene molecules incorporated in each RPA product as reflected in the SWV signals obtained (SWV raw data can be seen in Fig. S5).

3.3. Selection of the optimum percentage of ferrocene-labelled dNTPs for solid-phase RPA

This study was also carried out using solid-phase RPA amplification with fully complementary primer and target dsDNA and redox labelled oligonucleotides, and as can be seen in Fig. 2, hindering of the RPA reaction with increasing percentages of dN^{Fc}TP was more evident, which can be attributed to the fact that one of the primers is surface confined. In the case of both dC^{Fc}TP and dG^{Fc}TP, decreasing signals were observed at >60% labelled dNTPs, whilst dA^{Fc}TPs and dU^{Fc}TPs were well tolerated up to 90% and gave strong signals in 50–90% ratios. Apparently, the higher proportion of modified A-T pairs was better tolerated in the RPA process than modified G-C pairs (Fig. 2B and Fig. S6).

The denaturation step (glycine-HCl, pH 3) used following chemisorption of thiolated solution-phase RPA products was also employed here, serving to remove any of the RPA proteins adhered to the gold electrode surface or dN^{Fc}TP sequestered in the protein matrix or the double helix DNA. This is demonstrated by a decrease in charge transfer resistance in electrochemical impedance spectroscopy following denaturation (Fig. S7). As was observed with the solution-phase RPA, this denaturing step disrupted the rigidity of the surface bound dsDNA and facilitated considerable signal enhancement due to the flexibility of the ssDNA resulting in increased proximity to the electrode surface, providing improved electron transfer, which translated in higher specific signals. Whilst the signal is increased, this step is not absolutely essential and the assay can also be carried out without this denaturation step. However, we have observed that the assay reproducibility is improved when the denaturation step is included.

Whilst each of the dN^{Fc}TP could be used individually, the possibility of using a mixture of all four labelled dN^{Fc}TPs would avoid any target-sequence dependent problems e.g. an over/under-representation of

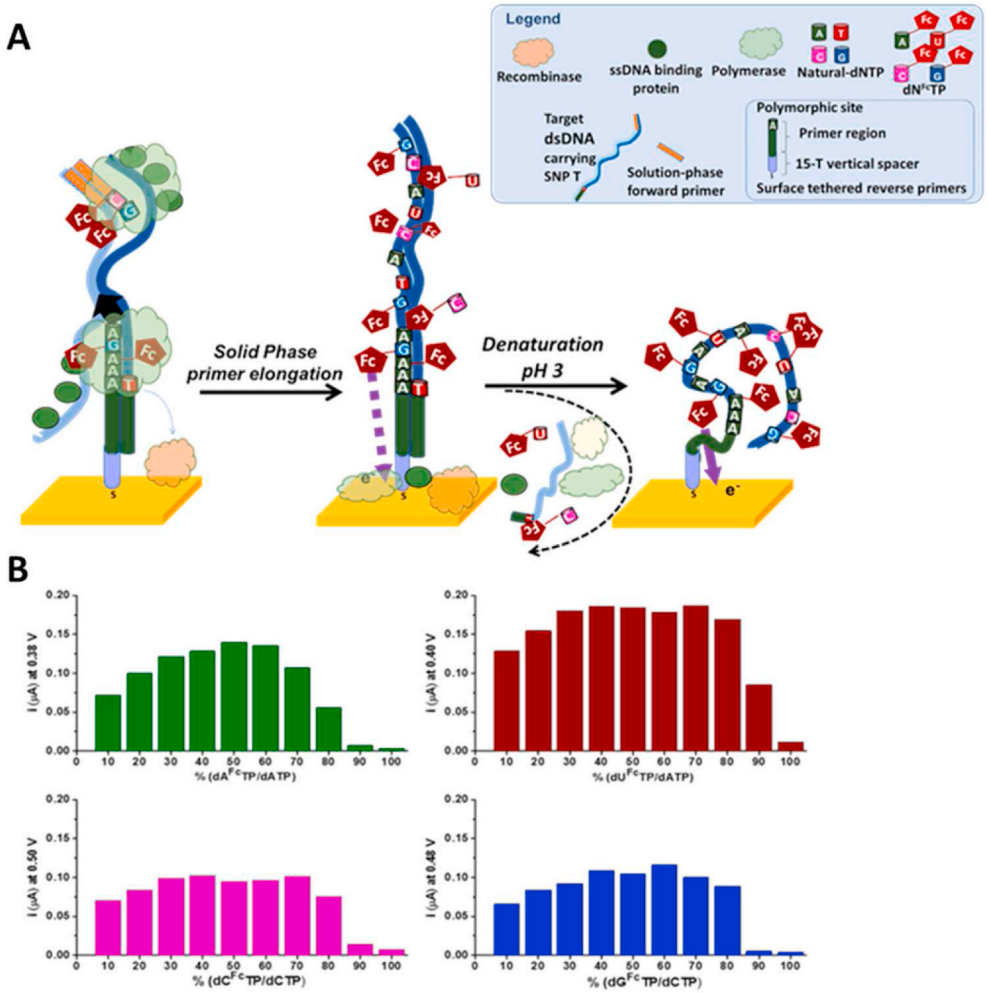

Fig. 2. (A) Schematic representation of the solid-phase RPA reaction, including the potential non-specific intercalation of dN^{Fc}TP in the double helix of amplified product and non-specific adsorption of the RPA proteins on the surface of electrode, which are effectively removed via the addition of the glycine-HCl, pH 3; (B) SWV peak intensities after SP-RPA using individual dN^{Fc}TP/dNTP at different percentages.

some bases in the target DNA. The four dN^{Fc}TPs and the four unmodified dNTPs (both at the same equimolar concentrations) were thus mixed at different percentages in the master mix, and as can be seen a maximal signal was observed with 30% of the equimolar dN^{Fc}TP mixture for solid-phase RPA, with the signal obtained being higher than any of those obtained with the individual dN^{Fc}TPs, due to an overall higher percentage of dN^{Fc}TPs being incorporated (Fig. 3). This higher overall level of incorporation can be attributed to an alleviation of steric hindrance facilitated by the elongating strand being able to incorporate labelled vs. unlabelled dNTPs as spatially preferred for each of the dNTPs.

3.4. Optimisation of the RPA conditions for SNP detection

Having demonstrated solid-phase RPA using dN^{Fc}TPs and optimising the percentages of dN^{Fc}TPs/dNTPs for each of the dNTPs as well as the combination of all four, the possibility to apply the approach of solid-phase primer elongation to single mismatch discrimination was pursued. The ability to differentiate between the complexes formed between the dsDNA target and each of the immobilised primers takes advantage of a slower elongation of mismatched sequences with respect to full complementarity. This differentiating effect can be magnified by controlling the reaction time as well as the concentration of the solution-phase forward primer and the concentration of $Mg(OAc)_2$, which is required to initiate the RPA reaction. In the absence of forward primer no amplification but only primer elongation occurs, which is evidenced in the significant decrease in the SWV signal with respect to when both primers are present in the RPA (Fig. S8).

To optimise these conditions, three electrode arrays were functionalised with primers with terminal A, T, C and G bases (Fig. S9). In the first iteration, 15 min RPA with 14 mM $Mg(OAc)_2$ was used following the manufacturer's recommendations, and the forward primer concentration was sequentially decreased, with the highest specificity achieved using 0.5 μM of this primer. A range of RPA reaction times (5–30 min) were tested, and 15 min observed optimum. Finally, the $Mg(OAc)_2$ concentration was evaluated, and 10 mM observed to be the best concentration for maximum SNP discrimination.

3.5. Comparison of SNP discrimination in solution and solid phases

Using these optimised conditions, primer elongation mediated by solution-phase and solid-phase RPA and 30% dN^{Fc}TPs/dNTPs was carried out with the four different primers and the same target dsDNA. As observed in Fig. S10A, when using native dNTPs, 5 min was adequate to visualise a significant band for the positive primer A by gel electrophoresis, with negligible signals obtained with the control primers, with increasing band intensities over time. Fig. S10B highlights the impact of the ferrocene moiety on the reaction rate, as no band was observed until 15 min of reaction when dN^{Fc}TPs were present in the master mix for solution-phase RPA. As can be seen clearly in Fig. S11, for solid-phase RPA, where the DNA amplification and detection were combined and carried out at the same electrode, the maximum SNP discrimination was observed at 15 min of RPA reaction time.

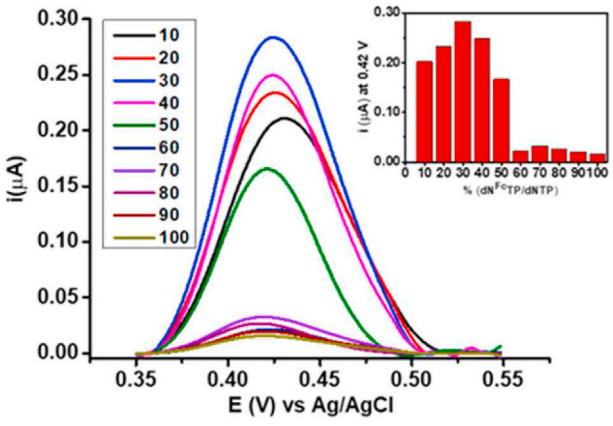

Fig. 3. Solid-phase RPA with all four dN^{Fc}TPs. SWV after SP-RPA using all four dN^{Fc}TPs/dNTPs at different percentages. The peak intensities vs. % dN^{Fc}TPs/dNTPs are shown in the inset.

As can be seen in Fig. 4A and B, whilst solution phase primer elongation did show enhanced primer elongation and an increased amplification for the primer with a terminal dATP (primer-A), a background signal was obtained for the other primers. Interestingly, in comparison, markedly improved discrimination was obtained using the solid-phase approach (Fig. 4C and D) where a clearly differentiable signal was obtained with a clearly defined peak observed for the primer-A and negligible peaks for primers-T/G/C. This can be attributed to the higher steric hindrance of the solid support as compared with the solution RPA enhancing the effect of the constraints imposed on the reaction, with this increased stringency resulting in higher specificity.

3.6. Application of the approach for SNP detection from fingerprick samples

The assay was finally exploited in a real-case scenario using fingerprick blood samples. Fingerprick blood samples were diluted in EDTA solution to avoid coagulation, heated for 30 s at 95 °C to extract DNA and, without further purification, mixed with the RPA master mix at different ratios. A first screening to elucidate the optimum ratio of the fingerpick blood sample with the RPA reagents was carried out using solution-phase RPA. As can be observed in Fig. S12, no amplification product was observed using a 1:2 dilution (blood: RPA master mix), probably due to the matrix effect of the blood. As can be seen in Fig. S12B, the intensity of bands increased when the ratio of the blood: RPA master mix was increased to 1:7, from where the amplification yield remained constant. This range of ratios (from 1:2 to 1:7 v/v, red square in Fig. S12B) were evaluated using solid-phase RPA and electrochemical detection (Fig. S12C). For the dilution 1:2, no reproducible SWV signals were obtained as illustrated in Fig. S12C and was discarded for the final approach while similar signal intensities with acceptable reproducibility were achieved for the rest of ratios. Finally, a dilution 1:3 blood:RPA reagents was selected for further solid-phase primer elongation experiments.

An optimisation of the assay time required for maximum SNP discrimination was carried out using the 1:3 blood:RPA ratio (Fig. S13A) and solid-phase combined with electrochemical detection (Fig. S13B). Fig. S13A shows that for solution-phase reaction, even the SNP was identified, the intensity of the band was very small as compared to that obtained for the synthetic target (Fig. S10B) at 15-min reaction time. This can be attributed to be due to either to the matrix effect or the lower accessibility of primers to the target region of the genomic DNA as compared with the shorter synthetic DNA, or a combination of both factors. Longer amplification times did not improve the results as primer elongation was observed for primers, thus preventing SNP identification.

For solid-phase RPA reaction (Fig. S13B), even though the SWV signals obtained are lower than those observed for the synthetic target (Fig. S11), the SNP detection was unequivocally achieved at 10 min, with the optimum differentiation being observed for 15 min primer elongation via solid-phase isothermal amplification.

Finally, the developed methodology with the electrode array housed within the microfluidics, was tested using eight different human fingerprick blood samples to demonstrate the potential of the approach for an eventual use at the point-of-care (Fig. 5 and Fig. S14). As the frequency of the SNP can have geographic variations, we selected different populations for this study (3 European, 3 Latin American and 2 Asian). The different frequencies of the alleles for each population can be found in Table S19.

Eight fresh fingerprick blood samples (10 μL) were collected using

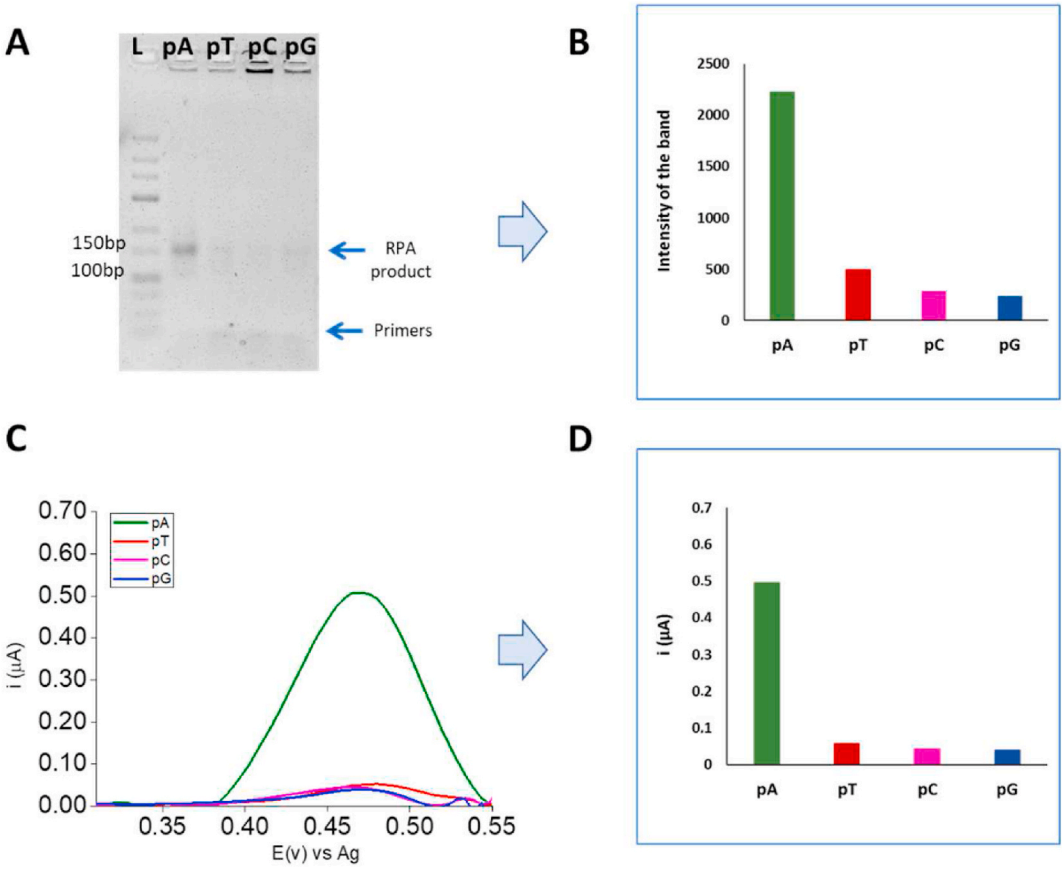

Fig. 4. SNP detection in a dsDNA target containing SNP T, prepared from synthetic ssDNA (Table S18), (pA - primer A-pT; primer T; pC- primer C; pG-primer G: L -Ladder). (A) Electrophoresis gel of RPA products amplified in solution; (B) corresponding intensities of the electrophoresis gel calculated by Image J software; (C) SWV of solid-phase approach and (D) The peak intensities of SWV of solid-phase approach. In all cases using 30% all four $dN^{Fc}TP$/dNTP at the optimum conditions.

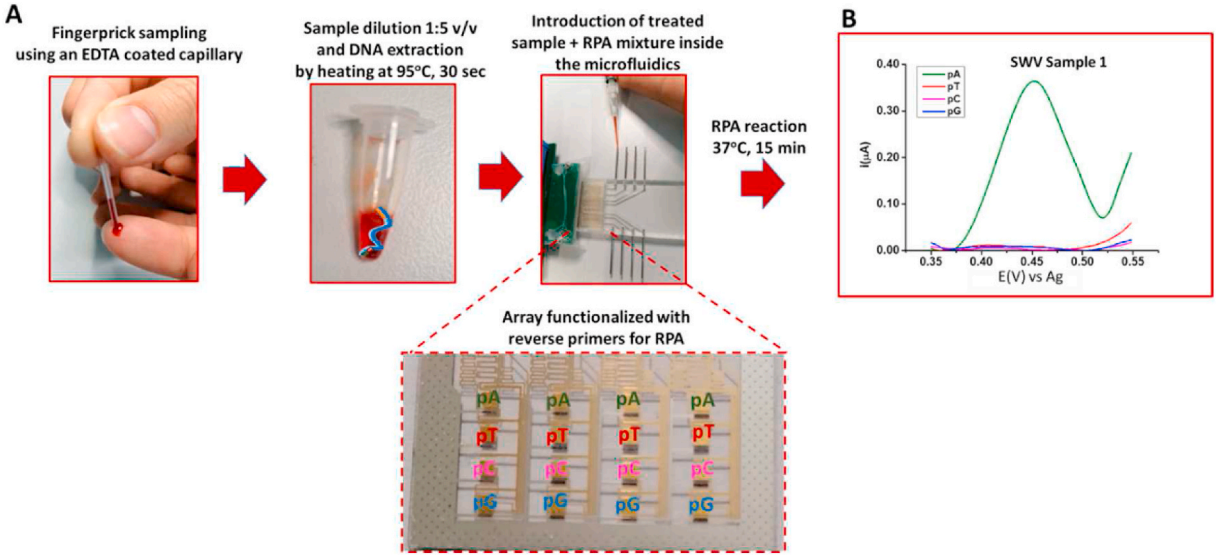

Fig. 5. SNP detection in real samples. (A) Flow through of SNP detection using fingerprick samples (pA - primer A-pT; primer T; pC- primer C; pG-primer G) and (B) SWV of RPA product amplified from a fingerprick blood sample.

EDTA coated capillaries to avoid coagulation, diluted 5 times in water and heated to 95 °C for 30 s to extract the DNA. The heated blood samples were then mixed with the RPA reagents and transferred to pre-functionalised electrode arrays placed in microfluidic housing. Following 15 min at 37 °C, the array was thoroughly washed with glycine pH 3 and re-filled with a solution of glycine and $Sr(NO_3)_2$ as supporting electrolyte. As expected, in all cases, the SNP detected was the wild type (T) found in the majority of the population, which was confirmed by Ion Torrent Next Generation Sequencing (see SM), thus demonstrating the rapid, reliable and easy identification of a SNP

directly from a fingerprick blood sample.

The genomic DNA from fingerprick samples was diluted and extracted with thermal lysis prior to be amplified with modified primers containing specific barcodes for the sequencing (Table S1). The data was analysed by filtering it to the expected size (80 bp) and collapsed to identify the most representative sequences which contains the SNP. The sequencing results corroborated that SNP T is the SNP found in the blood samples (Table S2 – Table S17).

Taking into consideration that there are between 4000 and 11,000 white blood cells per microlitre of blood, with two genome copies per cell, using a 10 μL fingerprick blood sample, SNP discrimination is possible at 8×10^5 copies of DNA, and presumably also at lower levels. Detecting a 130 bp fragment of the human genome, this translates to ca. 13.3 fM, demonstrating that the methodology can be used with femtomolar and it can be expected even lower concentrations of DNA, but in this work we are focused on the direct detection of the SNP in a fingerprick blood sample.

4. Conclusions

In summary, electrochemical detection of isothermal solid-phase primer elongation from gold electrode surfaces using redox-labelled oligonucleotides was exploited for the detection of the hypertrophic cardiomyopathy-associated single nucleotide polymorphism in the Myosin Heavy Chain 7 (MYH7) gene. The incorporation of each of the four labelled oligonucleotides ($dA^{Fc}TP$, $dC^{Fc}TP$, $dG^{Fc}TP$ and $dU^{Fc}TP$) as well as a mixture of all four ($d(A,C,G,U)^{Fc}TP$, $dN^{Fc}TP$) using isothermal recombinase polymerase amplification was initially demonstrated using solution-phase primer elongation, and the products analysed using gel electrophoresis. This optimisation of the ratio of labelled:unlabelled dNTPs was then carried out using solid-phase primer elongation from gold electrodes, with the best combinations elucidated to be (80% $dU^{Fc}TP$, 70% $dC^{Fc}TP$, 60% $dG^{Fc}TP$, 60% $dA^{Fc}TP$ and 30% x ($d(A,C,G,U)^{Fc}TP$). Fifteen minutes of the isothermal solid-phase primer elongation with 10 mM $Mg(OAc)_2$ was observed to give the maximum differentiation of the positive signal over the background. Fingerprick blood samples were procured, diluted and the white blood cells lysed by heating to 95 °C for 30 s and directly mixed with the RPA reagents containing 30% $dN^{Fc}TPs$, added to the electrode array and solid-phase primer elongation was carried out for 15 min at 37 °C. Square wave voltammetry confirmed elongation with the $dN^{Fc}TPs$ at just one electrode, corresponding to the primer with an A terminal base, indicative of T being present at the SNP site, which was correlated by next generation sequencing.

In many examples of SNPs in the human genome, it is only required to differentiate between two nucleotides – the wild type and the variant. Specifically in the model system used in this study, for hypertrophic cardiomyopathy T is the wild type and G is the variant, and the other 2 functionalised electrodes are used as negative controls to further demonstrate the reliability of the assay. The platform reported is generic platform and can find application in a plethora of applications where reliable identification of SNPs are required. Examples include the detection of antibiotic resistance where there can be more than two possible nucleotides at the SNP site, or in forensics where SNP analysis can provide phenotypic predictions as well as unique genetic fingerprints, often there can be four possible nucleotides at the SNP site. Further work is focusing on extending to simultaneous detection of multiple SNPs in fingerprick blood samples for diverse applications, such as the aforementioned antibiotic resistance, forensics, as well as further clinical applications.

CRediT authorship contribution statement

Mayreli Ortiz: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing. **Miriam Jauset-Rubio:** Data curation, Writing – original draft, Writing – review & editing. **David Kodr:** Data curation, Writing – original draft, Writing – review & editing. **Anna Simonova:** Data curation, Writing – original draft, Writing – review & editing. **Michal Hocek:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Ciara K. O'Sullivan:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2021.113825.

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