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ELECTROCHEMICAL PRIMER EXTENSION FOR THE DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS USING DIDEOXYNUCLEOTIDES LABELLED WITH POLYOXOMETALATES

MASTER'S DEGREE FINAL PROJECT

Supervisors: Dr. Ciara O'Sullivan and Dr. Mayreli Ortiz

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Abstract. Single Nucleotide Polymorphism (SNPs) are important genetic markers that can be helpful for identifying predisposition to a disease, patient stratification in the future paradigm of personalized medicine as well as an advanced forensic tool. Following identification of the location of SNPs using next generation sequencing technologies, primer extension reactions can be used for rapid identification of which SNP is present at the identified SNP site using dideoxynucleotides (ddNTPs) that will elongate a DNA double strand by just one base exactly at the SNP site. A novel approach of the PEX reaction previously introduced by our group is Electrochemical Primer Extension (EPEX), where dideoxynucleotides (ddNTPs) labelled with different electrochemical markers were tested. In this work, for the first time ddNTPs labelled with Keggin and Dawson polyoxometalates (POMs) are effectively incorporated using the EPEX reaction to elongate immobilized primers using the cardiomyopathy MYH7 gene as a model system. We successfully detected four different SNPs, representing each of the bases, using differential pulse voltammetry to detect the incorporated redox-labelled ddNTPs. The polymerase chain reaction was carried out to amplify a base pair region containing the four different bases, as a means of stimulating real patient samples. Once the target DNA products from asymmetric PCR were obtained, the single stranded DNA were tested in the EPEX reaction obtaining 100% of correct base incorporation and SNP detection for the Dawson POM. This study is an important development to achieve a cost-effective platform for detecting SNPs at identified locations.

Key words: Single Nucleotide Polymorphism, Dideoxynucleotides, Electrochemical Primer Extension, Polyoxometalates.

INTRODUCTION

Since the Human Genome Project (HGP) was completed in 2001, it has been possible to further understand the genetic causes of many diseases. To date, many strategies to treat and detect those diseases have arisen from the knowledge collected during the HGP, resulting in its contribution in medicine to be comparable to that of Mendeleev's periodic table in chemistry [1].

As was predicted, the detailed information about all the human genes that came as a consequence of the culmination of the HGP, facilitated the identification of mutations that cause or can be used to identify predisposition to multifactorial diseases [2] and as a natural consequence, there is a mature need to develop new genotyping methods and technologies that are throughput and accurate, allowing a better understanding of genetic and genomic data [1].

In the human genome sequence, there are individual variations such as insertions and deletions (indels), microsatellites (MSs) as well as single nucleotide polymorphisms (SNPs)[3]. The vast majority of genomic variations, around 90%, are determined by SNPs [3]. SNPs are single base pair mutations in a genome that occur in at least 1% of the total population, and they are expected to be crucial in the identification of genes that determine complex diseases [2] as well as for patient stratification for medicine prescription. By unraveling the human genome, it was found the presence of around 15 million SNPs, around 1 every 300-1000 bases [4].

The importance of SNP genotyping is that only by analyzing sequence variation in DNA, it can be reveal the risk to certain diseases [4], perform evolutionary studies in human populations [5], and provide information on drug metabolism [6]. As a result, it will facilitate a personalized medicine.

At present, next generation sequencing is being used to determine the location of SNPs in different populations

and identifying SNPs associated illnesses [7]. Next generation sequencing technology has evolved from first generation sequencing, the method proposed by Frederick Sanger in 1977 where dideoxynucleotides (ddNTPs) are exploited for a selective addition of the nucleotides in the DNA replication [8]. ddNTPs are modified nucleotides that lack the 2' and 3'-hydroxyl groups [9] (Figure 1), signifying, that after the ddNTP is incorporated in the DNA strand with a DNA polymerase, it will not be possible to add more nucleotides as; no more phosphodiester bonds can be formed due to the lack of hydroxyl group [9].

As a result, ddNTPs labelled to different types of markers, are very useful when trying to locate whether or not there is a point mutation in a gene, and genotyping methods using this molecules have been developed, including the Primer Extension (PEX) assay which was a method that was first used to identify the initiation site for a new gene. [10]

An evolution of the PEX assay was Arrayed Primer Extension (APEX), a genotyping method useful for testing known mutations on large genomic regions of DNA using fluorescently labelled dideoxynucleotides (ddNTPs) [11]. By using ligation or polymerase based elongation, a specific base complementary to that on the sequence under interrogation, (ddATP, ddGTP, ddTTP, ddCTP), is incorporated and this can be to search for single base mutations or SNPs [11].

One of the advantages of APEX is that it can be used to find multiple SNPs simultaneously in a multiplexed assay [12]. Nevertheless, some disadvantages of APEX include the need of robust instrumentation to detect the fluorescence signal, as well as advanced data processing, where there is the need for a specialized software to convert the fluorescence information into sequence data [13]. Furthermore, there are problems due to the instability of the fluorophore labels to bleaching, which enforces the use of antifade reagents in the process in order to achieve correct imaging [14].

With the objective of finding alternative strategies to detect SNPs, the use of electrochemical labels can be considered. In 1970 there was the first reported demonstration of the electrochemical oxidation of purine nucleobases at carbon electrodes [15]. This studies implied that DNA and nucleic acids in general, could be detected using electrochemical methods. In the 1990's, miniaturized nucleic acid-modified electrodes were developed, facilitating a better sensitivity for the detection of nucleic acids and making these approaches competitive in comparison to standard molecular biology methods [15].

With that focus, our group developed a variation of the Arrayed Primer Extension (APEX), replacing the fluorescently labelled dideoxynucleotides used in APEX, with dNTPs functionalized with redox labels in Electrochemical Primer Extension (EPEX) [16]. That study demonstrated the successful incorporation of single nucleotides modified with conventional electrochemical markers such as ferrocene and methylene blue that bare different redox potentials, which allowed electrochemical differentiation between each signal to detect different SNPs.
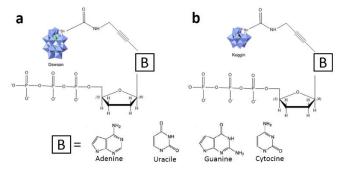

Figure 1. The redox active dideoxy nucleotide triphosphates used for electrochemical primer extension assays. Keggin POM and Dawson POM (-0.77 V) Potential vs. Ag/AgCl.

Electrochemical Primer Extension has advantages over its array-based predecessor, such as its cost-effectiveness, portability and vastly less complex instrumentation [16]. Still, conventional electrochemical markers have some issues that need to be overcome, including ferrocene oxidation [17], as well as the ability of methylene blue to both act as an intercalador as well as electrostatic molecular interactions with guanines [18].

To overcome this, the use of new electrochemical markers is of interest for functionalization of DNA primers and nucleotides. Polyoxometalates (POMs) are anionic metal oxygen clusters that are known to have
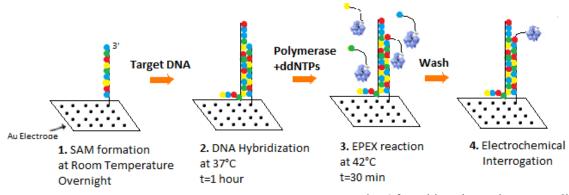
Figure 2. Electrochemical Primer Extension Protocol

remarkable properties that add value to their use, such as tunable redox properties and magnetism [19]. This molecules are formed from early transition metals (such as W, Mo or V), oxygen, and can also bear a heteroatom (Si, P, Ge, etc) [19] (Figure 2). A special added value property of POMs is that they have been demonstrated to be biocompatible, having been successfully bioconjugated with DNA primers [20] and thus, have potential for use in electrochemical SNP detection.

In this study, for the first time, and to the best of our knowledge we will use ddNTPs labelled with polyoxometalates and perform an EPEX reaction in order to detect SNPs, using MYH7 cardiomyopathy gene as a model system. We also perform specificity studies of the ddNTP-POMs when mixed in a solution with other electrochemical markers, and optimize the reaction conditions such as the polymerase enzyme used and perform a comparison between the signals obtained by different kind of POMs. Finally, we applied the developed technique to the multiplexed detection of SNPs on an assymetric PCR generated amplicon mimicking the final use of their approach on real patient samples.

EXPERIMENTAL PART

1. Electrode cleaning: Gold electrodes were used to perform the experiments. First, a mechanical cleaning is carried out. The bar gold electrodes are polished with 0.3 μm alumina powder for 5 min followed by washing thoroughly with MilliQ water and sonication first in MilliQ water and then in pure ethanol for 5 min minutes

each. After this, electrodes are polished in 0.05 μm alumina for 5 min and the last procedure repeated, by washing thoroughly with MilliQ water followed by sonication first in MilliQ water and then in pure ethanol for 10 minutes each. Secondly, an electrochemical cleaning is performed with 0.5 M H_2SO_4, sweeping for 50 cycles between 0.2 and 1.6 V vs. Ag/AgCl reference electrode. In order to check the suitability of the electrodes after cleaning, a Cyclic Voltammetry test is performed. In this test, the electrodes are considered clean if the peak separation between the maximum and minimum peak in the voltammogram is 100 mV or less.

2. Electrochemical Primer Extension Protocol: The proposed protocol for electrochemical primer extension is based on the Arrayed Primer Extension (APEX) reaction. The APEX reaction is isothermal and basically, consists of detection of the SNP by having a DNA probe surface tethered on an array, followed by addition of the complementary DNA target containing the SNP that is being interrogated [14] (Figure 3). The DNA target and probe hybridize because of their complementarity. A polymerase enzyme is then added, together with electrochemically labelled ddNTPs to complete the single base extension reaction.

2.1. Self-Assembly Monolayer (SAM) Formation: The surface chemistry for the SAM formation is based on the use of a dithiol modified single stranded DNA on gold. The cleaned gold electrodes were immersed in a solution of 1 μL of 5 μM thiol/thioctic acid modified ssDNA (probe), 0.1 μL DT1 (10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol) was used to co-immobilize and mixed with 18.5 μL KH_2PO_4. After 3h of incubation at room temperature the electrodes were rinsed with Tris buffer [10 mM Tris

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buffer with 0.5 M NaCl solution (pH 7)] and dried with a nitrogen stream.

2.2. DNA hybridization: The hybridization of 1 μM target DNA was performed by incubation of the prepared electrodes in 30 μL of buffer solution [10 mM Tris buffer with 0.5 M NaCl solution (pH 7)] each solution containing the respective target DNA and using individual electrodes for each of the bases. Incubation was performed for 1 h at 37°C and as a result, it formed the complementary duplex up until SNP site. The electrodes were washed with the hybridization buffer, dried with argon, and were ready for the primer extension (PEX) reaction.

2.3. EPEX reaction: The reaction of nucleotide incorporation is performed by adding 0.5 μL of a polymerase enzyme, 1 μM of electrochemically labelled ddNTP and 20 μL of enzyme reaction buffer. The solution is incubated on the previously prepared electrodes for 30 min at 42°C. For this nucleotide incorporation reaction two sets of experiments were designed by varying two parameters: the polymerase enzymes, and the labelled dideoxynucleotides (ddNTPs).

2.4. Electrochemical interrogation: DPV was used to detect the incorporation of the labeled ddNTPs. The measurements were performed at room temperature in a 10 mL electrochemical cell with a three electrode configuration having the gold electrode as the working electrode (with inner diameter of 2 mm), a platinum wire as the counter electrode and an Ag/AgCl electrode as the reference electrode. The electrodes were transferred to 10 mM Tris buffer containing 0.5 M NaCl, pH7. DPVs were recorded at various potential windows depending on the redox potential of the labels (vs Ag/AgCl), pulse amplitude of 0.1 V, step potential of 10mV, pulse width 100 ms and pulse period 5 ms. All 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).

3. EPEX optimization Experiment: In order to identify the optimal buffer pH and polymerase enzyme for the

EPEX protocol, the reaction is tested varying these two parameters.

Tris buffers at acid, neutral and basic pH are tested. Solutions containing pH 3, 7 and 8 are prepared by adjusting the pH of the original buffer (pH 8) using 1 M hydrocloric acid. The prepared electrodes were incubated with a solution containing 1 μM of the redox labelled ddNTP, 20 mM Tris solutions in the three different pHs, enzyme buffer containing 50% v/v glycerol, 0.1 mM EDTA, 0.5% v/v Tween, 0.5% v/v nonidet-P40, 1 mM DTT, 100 mM KCl, 65 mM $MgCl_2$, and 0.5 units of Thermosequanase® DNA polymerase. The electrodes were sealed with parafilm, incubated at 42°C for an additional 30 min, subsequently washed with hybridization buffer, and electrochemical detection carried out.

In a second experiment, once the most suitable buffer was chosen for the EPEX reaction, polymerase enzyme variation experiments were performed. Thermosequenase® enzyme was used in one experiment in the conditions proposed by Mehdi et al [16] for the electrochemical primer extension, at 42°C. 20 μL of 1X Therminator® Buffer containing 50% v/v glycerol, 0.1 mM EDTA, 0.5% v/v Tween, 0.5% v/v nonidet-P40, 1 mM DTT, 100 mM KCl, 65 mM $MgCl_2$ were added to start the reaction. The electrodes were sealed with parafilm, incubated at 42°C for an additional 30 min, subsequently washed with hybridization buffer, and kept ready for electrochemical assay. The Therminator® enzyme was also tested for the incorporation of electrochemically labelled ddNTPs at three different temperatures: 75°C, which is the proposed working temperature for the enzyme, 50°C, and 42°C which is the same temperature previously optimized and used in Thermosequenase® (16). 20 μL of 1X ThermoPol® reaction buffer containing 20mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100 was used. The electrodes were sealed with parafilm, incubated at the three different temperatures (75°C, 50°C and 42°C) for an additional 30 min, subsequently washed with hybridization buffer, electrochemical detection carried out.

4. Non-functionalized POMs' Stability*:* Solutions of pure Dawson and Keggin POMs (Figure 3) were prepared by mixing 6.053mg of Dawson POM per 1ml

of MilliQ water to make a 1mM solution, and 4.079 mg of Keggin POM per 1ml of MilliQ to make a 1 mM solution. 50 µL of each solution were mixed with 50 µL of Tris buffer pH 7 in order to measure the electrochemical response of the POMs and their stability by measuring every 10 minutes. DPVs and cyclic voltammetries were performed in order to find the oxidation and reduction peaks in solution.
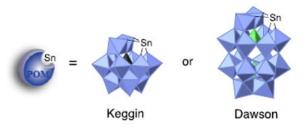
5. ddNTP-POM EPEX reaction: ddNTPs (ddATP, ddGTP, ddTTP, ddCTP) functionalized with D POM and K POM are used in the reaction of nucleotide incorporation. The reaction is performed using 0.5 µL of Therminator® polymerase enzyme, 1 µM of electrochemically labelled ddNTP and 20 µL ThermoPol® reaction buffer. The solution is incubated in the previously prepared electrodes for 30 min at 42°C.

6. ddNTP-POM EPEX reaction in a long target sequence: In this experiment, the target sequence used in the hybridization step is a 100 nucleotide long (100bp) single stranded DNA sequence. The hybridization is performed using the same conditions described (1hr at 37°C) and after this, similarly to the previous experiment, ddNTPs (ddATP, ddGTP, ddTTP, ddCTP) functionalized with D POM and K POM are used in the reaction of nucleotide incorporation. For the EPEX reaction, 0.5 µL of Therminator® polymerase enzyme, 1 µM of electrochemically labelled ddNTP and 20 µL ThermoPol® reaction buffer are added. The solution is incubated in the previously prepared electrodes for 30 min at 42°C.

7. ddNTP-POM Specificity Studies: ddNTPs (ddATP, ddGTP, ddTTP, ddCTP) functionalized with D POM and K POM are mixed with ddNTPs functionalized with ferrocene, anthraquinone and phenothiazine (ddATP-

AQ, ddGTP-Fc, ddCTP-PTZ). A total set of eight EPEX reactions was performed by mixing 1 µM ddNTP-K or ddNTP-D with two different electrochemically labeled bases (1 µM each), using non-specific ddNTPs for each POM-ddNTP; (e.g. ddATP-K was mixed with ddGTP-Fc and ddCTP-PTZ) in the presence of 0.5 µL of Therminator® polymerase enzyme, and 20 µL ThermoPol® reaction buffer. The solution is incubated on the previously prepared electrodes for 30 min at 42°C.

7. PCR amplification of DNA long target for further SNP detection in the EPEX reaction: The 100-base oligonucleotide DNA target is a synthetic sequence designed to emulate the SNP containing regions of the MYH7 gene. A master mix was prepared using 30 µL PCR buffer, 30 µL enhancer, 15 µL dNTPs, 3 µL forward primer, 3 µL reverse primer, 0.6 µL Kappa enzyme and the volume was completed to 150 µL with MilliQ water.

7.1. PCR Optimization: The master mix was divided in 6 parts to perform a PCR optimization. To three of the master mix samples 1.5 µL of 1 nM target DNA was added to be amplified for a different number of cycles (5, 10 and 15 cycles) and the remaining three samples were used as a negative control without any addition of target DNA. The PCR program used was one cycle of denaturation at 95°C for two minutes, followed by annealing at 58°C for 30 seconds and then 72°C elongation for 30 seconds for 5, 10 or 15 cycles depending on the sample, followed by a final extension step was performed at 72°C for 5 minutes. The PCR products were analyzed using gel electrophoresis in 2% w/v agarose gel in acetate buffer, in order to select the optimal amount of cycles.

7.2. Asymmetric PCR: 10 µL of the optimized PCR product were taken and added to a new master mix containing 10 µL PCR buffer, 10 µL enhancer, 5 µL dNTPs, 4 µL forward primer, 0.2 µL Kappa enzyme and the volume was made up to 50 µL with MilliQ water. In
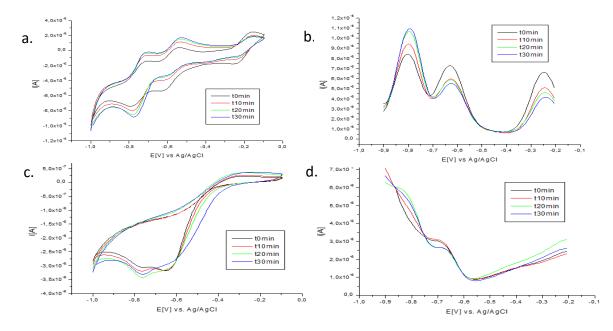
Figure 4. a) Cyclic Voltammogram of the Non-functionalized pure Dawson POM b) Differential Pulse Voltammogram of Non-functionalized pure Dawson POM c) Cyclic Voltammogram of the Non-functionalized pure Keggin POM d) Differential Pulse Voltammogram of Non-functionalized pure Keggin POM

this case, only an excess of forward primer is needed and no reverse primer is used in order to favour the generation of single stranded target DNA. The PCR program used was 12 cycles, with denaturation at 95°C for two minutes, annealing at 58°C for 30 seconds and elongation at 72°C for 2 minutes and a final an extension step at 72°C for 5 minutes. The PCR products were analyzed using gel electrophoresis in 2% w/v agarose gel in acetate buffer, in order to confirm the generation of the single stranded target DNA.

7.3. PCR products digestion and purification: A digestion of the products obtained in the Assymetric PCR was performed by adding 2 µL of lambda exonuclease enzyme and 10 µL of exonuclease buffer in a PCR master mix like the one previously described. The samples were digested in a Biorad iCycler at 37°C for 2 hours followed by a denaturation of the enzyme at 80°C for 10 minutes. After this, an Oligo Clean & Concentrator™ kit was used to purify the products and a final gel electrophoresis was performed in 2% w/v agarose gel in acetate buffer, to confirm the generation of single stranded DNA.

RESULTS & DISCUSSION

In order to achieve the best possible experimental conditions, before performing the primer extension experiments two different enzymes were tested; Thermosequanase® (GE Healthcare) has been widely used for many PEX experiments [12] [21] [22] and has been demonstrated to work for single base elongation at 42°C [16] Its cost is 592€ per 1000 units. In order to achieve cost-effective molecular tools, it was highly desirable to work with a less expensive enzyme to perform the PEX reactions. Therminator® is a thermostable polymerase enzyme that has the ability to incorporate modified dideoxynucleotides in single base extensions. Its cost is 388€ per 1000 units. Since Therminator's proposed working temperature is 75°C, PEX experiments were performed at different temperatures (42°C, 50°C and 75°C) to probe if the base incorporation could take place at 42°C. We had a special interest in performing the reaction at this temperature, because the melting temperature of the oligonucleotides used in the experiments is 60°C. Therminator® enzyme was demonstrated to incorporate electrochemically labeled ddNTPs at the three tested temperatures. In comparison with Thermosequenase® enzyme, the electrochemical signal observed following incorporation with the Therminator® at 42°C was even improved,
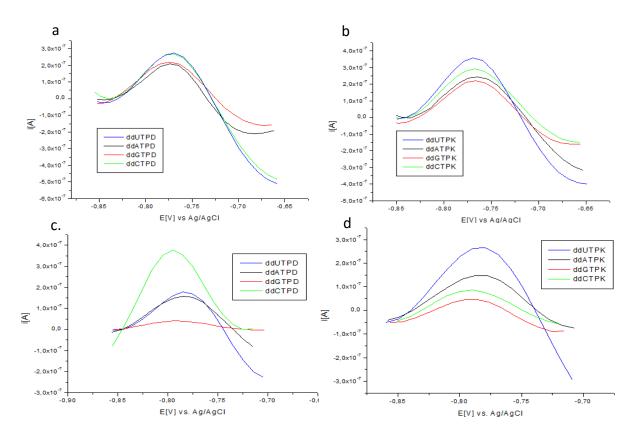
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Figure 5. a) Differential Pulse Voltammogram for the ddNTP-Dawson POM b) Differential Pulse Voltammogram for the different ddNTP-Keggin POM. Each ddNTP-POM was incorporated in an individual reaction dependent on the specific base present at the SNP site. c) Differential Pulse Voltammogram for the different ddNTP-Dawson POM incorporated in a long target sequence d) Differential Pulse Voltammogram for the different ddNTP-Keggin POM incorporated in a long target sequence

demonstrating Therminator® to be suitable for further experiments with POM labeled ddNTPs.

The EPEX reaction was performed in different pH buffers (pH 3, 7 and 8); it was found that neutral buffers produce the narrowest and clearest DPV peak. Basic buffers destabilize the POMs clusters and produce a different electrochemical response. Since POMs are metal clusters, they can be very sensitive to basic pH, causing their disaggregation. Due to this, Tris Buffer pH 7 was used to perform the consequent PEX experiments in order to prevent any condition that could alter POM stability. A neutral buffer is also desired for our experiments since this is the physiological pH and optimum for DNA hybridization and ddNTP incorporation.

In order to further explore the stability of POMs, we performed cyclic voltammetries and differential pulse voltammetries on non-functionalized, pure Keggin and Dawson POMs solutions in pH 7 Tris Buffer (Figure 4). The figures show that the electrochemical signals are stable over time pH 7 solutions.

The DPVs show the reduction peaks, which correspond to the reduction of the tungsten moiety. For the Dawson POM (Fig. 4b) the peak values are -0.63 V vs Ag/AgCl and -0.79 V vs Ag/AgCl and for the Keggin POM (Fig. 4d) the first peak appears at -0.83 V vs Ag/AgCl, but is more visible at $t = 20\ min$ than in the other times, while the second peak appears at -0.68 V vs Ag/AgCl for $t = 0\ min$ and $t = 10\ min$ and at -0.66 V vs Ag/AgCl for $t = 20\ min$ and $t = 30\ min$. This difference can be due to the POM chemistry, where the redox processes depend on the solvent and the protonation state [19]. Even though the pH of the buffer used in this study was pH 7, the results obtained are aligned to the ones obtained by Pope et al. (-0.647 and -0.830 V vs Ag/AgCl at pH 2.9) [23] and Debela et al. (-0.64 and -0,85V versus Ag/AgCl at pH 3,2) [19] and once again, the differences in the values are due to the difference in the pH that we used, which was of our interest due to the biological application of SNP detection that we want to develop in this study. This

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study also shows that POMs are stable at physiological pH.

Figure 5a and 5b show the electrochemical detection of the ddNTPs labeled with Keggin and Dawson POM for the four different bases of DNA. Each signal corresponds to an incorporation of the labeled complementary base specifically in the target SNP region. To the best of our knowledge, this is the first report of SNP detection with a POM electrochemical signal. For both Keggin and Dawson POM the DPV peak is at almost the same potential which is 0.769 V vs Ag/Ag/Cl for Keggin POM and 0.779 V vs Ag/AgCl for Dawson POM. In comparison with the reduction values obtained in the non-functionalized pure POMs (-0.63 V and -0.79 V vs Ag/AgCl for the Dawson POM and approximately -0.83 V and 0.67 V for the Keggin POM), this value lies between the two reduction potentials and does not correspond exactly to one of the two reduction peaks previously obtained. This is an expected value, since the ddNTP-POM is a nucleotide holding a modification, and is not exactly the same molecule as a non-functionalized POM. Moreover, it is incorporated in a much larger molecule which is a surface tethered DNA, and so, the molecular interactions of the POM may vary, causing a difference in the electrochemical behavior.

Once the incorporation of the ddNTP-POM Keggin and Dawson was demonstrated for a short oligonucleotide target sequence, the possibility of carrying the EPEX reaction using long synthetic target sequences, as well as PCR generated amplicons was tested.

The EPEX reaction using long target sequences was also demonstrated to successfully incorporate the base base I the correct SNP site, which is also detected with differential pulse voltammetry (Figures 5c and 5d). The voltammograms present some differences in comparison with the ones of short sequences. The intensity of the signal varies both for Keggin and Dawson POM. In the case of the Dawson POM the value of the potential shifts for the ddUTP and ddATP. However, the POM signal is strong enough to be detected electrochemically.
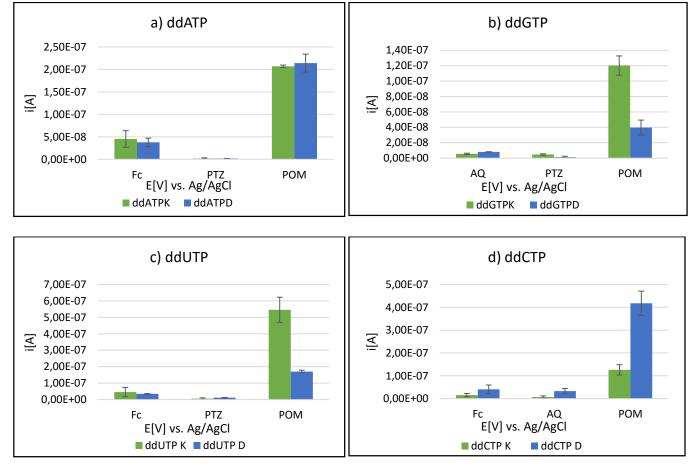

Figure 6. a) Differential Pulse Voltammogram for the different ddNTP-Dawson POM b) Differential Pulse Voltammogram for the different ddNTP-Keggin POM. Each ddNTP-POM was incorporated in an individual reaction dependent on the specific base present at the SNP site.

In order to study the specificity and selectivity of the nucleotide incorporation, the EPEX reaction was performed in a mixture of different redox labeled ddNTPs together with the specific POM labeled ddNTP complementary to the SNP base. The results for each POM-ddNTP studied showed that the DPV signal is significantly higher for the incorporated ddNTP-POM in comparison with the signals of the non-specifically labelled ddNTPs. This results confirm the specificity of the polymerase enzyme and its proofreading capacity to incorporate the correct modified ddNTPs.
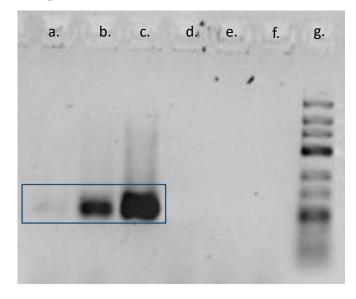

Figure 8. Single stranded target DNA generated after purification process a) Ladder b) Single Strand c) Comparison with 10 Cycle PCR

Figure 7. PCR optimization a) 5 Cycles b) 10 cycles c) 15 cycles d) 5 Cycles Blank e) 10 Cycles blank f) 15 cycles blank g) Ladder

Finally, polymerase chain reaction experiments were performed with the aim of testing the EPEX reaction with the generated DNA target products. A pilot PCR for optimization (Figure 7), showed that 10 PCR cycles produced enough double stranded DNA target product. Subsequently a combination of asymmetric PCR with exonuclease digestion was used to generate single stranded DNA for hybridization to the surface immobilized probe. As can be seen in Figure 8, the purified asymmetric DNA amplicons were efficiently generated.

In the case of the Dawson labeled ddNTPs, each was correctly incorporated at each designed SNP site and good DPV signals observed. However, in the case of Keggin POM labeled ddNTPs only ddUTP and ddGTP
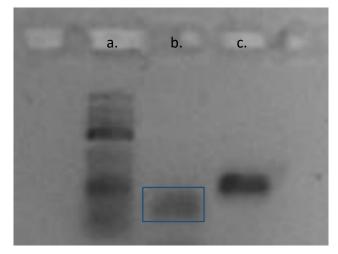
bases were observed to be incorporated. This results suggest a better behavior of the functionalized Dawson POM in PCR products but cannot be easily explained
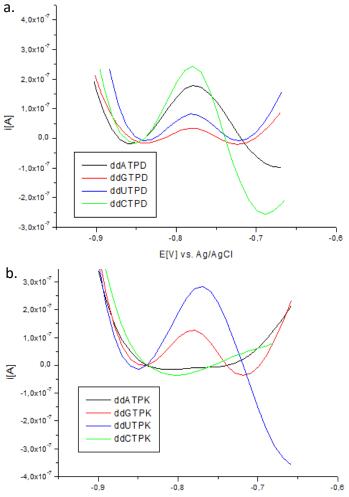

Figure 9. Differential Pulse Voltamogram for the detection of SNPs in target PCR product with ddNTPs labelled with a) Dawson POM b) Keggin POM

and further work will focus on optimization of the asymmetric PCR to generate higher levels of product for interrogation.

CONCLUSIONS

In summary, we have demonstrated for the first time the successful primer extension of ddNTPs labeled with Keggin and Dawson POMs, and their application in the electrochemical detection of SNPs. We also showed that the Therminator® enzyme is suitable for performing these kind of reactions, representing a less expensive alternative to the conventionally used Thermosequenase® enzyme.

This study provides the proof-of-concept that POMs can be used as electrochemical markers for detection of single nucleotide polymorphisms. It was demonstrated that POM labelled ddNTPs incorporate correctly in the SNP region of longer target sequences, and moreover, when mixed with other electrochemically labelled ddNTPs, the EPEX reaction performed using long target sequences was shown to be specific.

Finally, when the EPEX performed with the generated PCR target products, the base incorporation was shown to be better for Dawson POM, but the asymmetric PCR reaction requires further optimization.

As a future work, the electrochemical primer extension (EPEX) reaction using POMs has the potential to detect any type of SNPs, further experiments in real samples should be tested as well as a multiplex approach to detect several SNPs at the same time.

AKNOWLEDGMENTS

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