Biosensors and Bioelectronics 117 (2018) 201–206

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Electrochemical primer extension based on polyoxometalate electroactive labels for multiplexed detection of single nucleotide polymorphisms

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ARTICLE INFO

Keywords:
SNP detection
Polyoxometalate-labelled ddNTPs
Electrochemical primer extension reaction (éPEX)
Multiplexed electrochemical detection

ABSTRACT

Polyoxymetalates (POMs) ($[SiW_{11}O_{39}\{Sn(CH_2)_2CO)\}]^{4-}$ and $[P_2W_{17}O_{61}\{Sn(CH_2)_2CO)\}]^{6-}$) were used to modify dideoxynucleotides (ddNTPs) through amide bond formation, and applied to the multiplexed detection of single nucleotide polymorphisms (SNPs) in an electrochemical primer extension reaction. Each gold electrode of an array was functionalised with a short single stranded thiolated DNA probe, specifically designed to extend with the POM-ddNTP at the SNP site to be interrogated. The system was applied to the simultaneous detection of 4 SNPs within a single stranded 103-mer model target generated using asymmetric PCR, highlighting the potential of POM-ddNTPs for targeted, multiplexed SNP detection. The four DNA bases were successfully labelled with both ($[SiW_{11}O_{39}\{Sn(CH_2)_2CO)\}]^{4-}$ and $[P_2W_{17}O_{61}\{Sn(CH_2)_2CO)\}]^{6-}$), and $[SiW_{11}O_{39}\{Sn(CH_2)_2CO)\}]^{4-}$ demonstrated to be the more suitable due to its single oxidation peak, which provides an unequivocal signal. The POM-ddNTP enzymatically incorporated to the DNA anchored to the surface was visualised by AFM using gold coated mica. The developed assay has been demonstrated to be highly reproducible, simple to carry out and with very low non-specific background signals. Future work will focus on applying the developed platform to the detection of SNPs associated with rifampicin resistance in real samples from patients suffering from tuberculosis.

1. Introduction

Polyoxometalates (POMs) are anionic metal oxygen clusters with remarkable properties including tuneable redox properties, magnetism and biocompatibility (Boglio et al., 2008; Debela et al., 2014). POMs are formed from early transition metals (such as W, Mo or V), oxygen, and can also bear a heteroatom (Si, P, Ge, etc) (Boglio et al., 2008; Debela et al., 2014). Recently the successful bioconjugation of polyoxotungstates $[SiW_{11}O_{39}\{Sn(CH_2)_2CO)\}]^{4-}$ (SiW_{11}Sn-Keggin) and $[P_2W_{17}O_{61}\{Sn(CH_2)_2CO)\}]^{6-}$ (P_2W_{17}Sn-Dawson) with DNA primers (Debela et al., 2015), and deoxynucleotide triphosphates (Ortiz et al., 2017) and the incorporation of these POM-primers and POM-dNTPs in PCR has been described. In both cases, the electroactive properties of POM allowed rapid and sensitive electrochemical detection of a DNA target. In the work reported here, we wish to expand the extraordinary possibilities of POMs in electrochemical genotyping, by their application in SNP detection through an electrochemical primer extension (éPEX) reaction (Fig. 1).

A SNP is a DNA sequence variation occurring when a single nucleotide in the genome differs between members of a species or paired chromosomes and occur in at least 1% of the total population (Pastinen et al., 1997; Sachidanandam et al., 2001). By unravelling the human genome, and in subsequent international efforts such as the HapMap project, the presence of around 15 million SNPs has been identified to date (Durbin, 2001). SNP genotyping can be used to facilitate patient stratification, to detect predisposition to certain diseases (Durbin, 2001), to perform evolutionary studies (Hacia, 1999), as well as providing information on drug metabolism (Evans and Relling, 1999). Furthermore, SNP genotyping shows great promise for forensics, where SNPs can be used to obtain a genetic fingerprint even in degraded DNA samples, and can also be used for prediction of phenotypic characteristics. Whilst next generation sequencing has been extensively used for the identification and detection of SNPs, there is a defined need for the targeted detection of a specific subset of SNPs, rather than a genome wide SNP map.

The discrimination of a single point mismatch in DNA analysis is a

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https://doi.org/10.1016/j.bios.2018.06.014
Received 9 April 2018; Received in revised form 4 June 2018; Accepted 6 June 2018
Available online 07 June 2018
0956-5663/

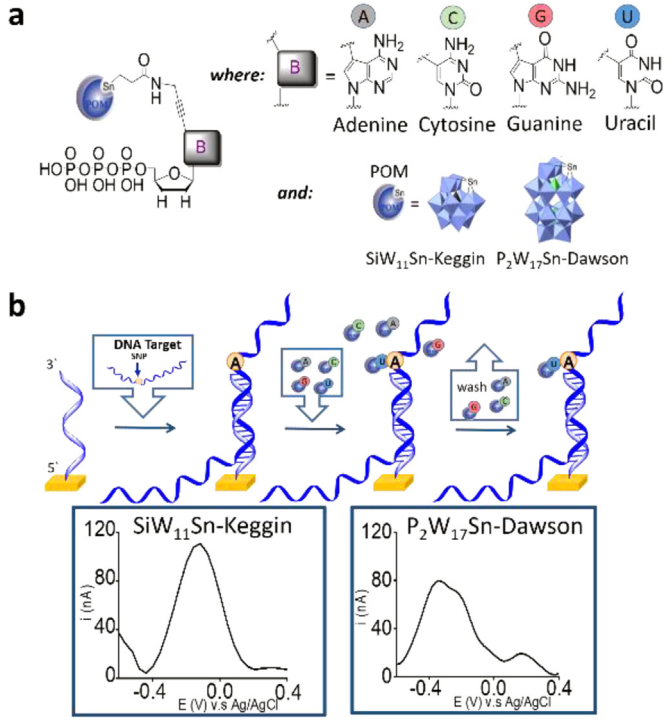

Fig. 1. Schematic representation of a) POM-modified-ddNTPs and b) the application of POM-modified-ddNTPs in ePEX (Insets: Differential Pulse Voltammograms (DPV) of $SiW_{11}Sn$-ddUTP and $P_2W_{17}Sn$-ddUTP incorporated at 3′-end of primers. DPV were recorded in 10 mM Tris + 0.5 NaCl, pH 7).

challenge that needs to be addressed, with multiplexed detection of SNPs finding increasing application in diagnostics, theranostics, as well as in advanced forensics. The combination of fluorescence and nanostructured composites have been demonstrated to be a highly promising strategy for the solution-based detection of SNPs (Tian et al., 2015a, 2015b; Yang et al., 2015; Duan et al., 2009). However, this approach suffers great difficulties in the simultaneous multiplexed detection of SNPs and to address this shortfall, array based primer extension (APEX) using fluorescent detection has been successfully employed. However, fluorescence APEX is inherently expensive and laboratory based due to the optics and instrumentation required. In the APEX reaction, an isothermal reaction that takes advantage of immobilised single stranded DNA (ssDNA) probes designed to hybridise to a single stranded PCR amplicon one base downstream from the SNP site being interrogated. Following hybridisation, labelled ddNTPs, modified nucleotides that lack the 2′ and 3′-hydroxyl groups, are added (Tonisson et al., 2010). Using either ligation or elongation with a ligase or polymerase enzyme, respectively, the immobilised probe is extended by a single labelled ddNTP, complementary to the SNP being addressed (Sanger et al., 1977) (Fig. 1b). Following incorporation of the ddNTP, no further phosphodiester bonds can be formed due to the lack of the 2′/3′-hydroxyl group, and thus further elongation cannot take place (Sanger et al., 1977).

Electrochemical techniques for screening of SNPs offer cost-effective and facile detection platforms as an alternative to colorietry (Zhang et al., 2018), fluorescence (Sanger et al., 1977) or radioactive detection (Krjutskov et al., 2008). A broad spectrum of electroactive markers such as intercalators (Wakai et al., 2004), ferrocene modified probes (Yu et al., 2001) and nanoparticle probes (Liu et al., 2007) and nanocrystal labelled nucleotides (Liu et al., 2005) have been reported for the electrochemical detection of SNPs. Solid phase ePEX is a variant of APEX using an electroactive label. In this context, we recently demonstrated the feasibility of performing ePEX reaction with ddNTPs labelled with different electroactive markers: ferrocene, anthraquinone, phenothiazine and methylene blue (Debela et al., 2016). Whilst the concept of ePEX was successfully demonstrated, a small level of non-specific background signal was observed due to the electrostatic interaction with methylene blue, and to this end, an alternative redox label was pursued. Consequently, we explored the possibility of using POMs to label ddNTPs for use in ePEX. POMs are robust molecules at neutral-acid pH, inexpensive and easy to prepare and functionalise, and offer a potential window compatible with biomolecules. Furthermore, we expanded on our previous proof-of-concept work, extending to simultaneous, multiplexed detection of SNPs using an electrode array housed within a microfluidic set-up.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade and used as received. Potassium dihydrogen phosphate (KH_2PO4), sodium chloride (NaCl), potassium chloride (KCl), were purchased from Fluka, while sodium perchlorate ($NaClO_4$) was received from Acros Organics. Sodium hydroxide, sulphuric acid (95–97%), Tris-HCl and boric acid were purchased from Scharlau, Barcelona, Spain and hydrochloric acid (35% v/v) from Panreac. Phosphate-buffered saline (PBS), trisodium citrate, acetone, dimethyl sulfoxide (DMSO), perchloric acid (70% v/v), triethylamine, glacial acetic acid, phenothiazine, anthraquinone carboxylic acid, ferrocene carboxylic acid, N-(3-dimethylaminopropyl N′-ethyl-carbodiimide hydrochloride and N-hydroxy succinimide were all purchased from Sigma Aldrich, Spain. The propargyl amino modified ddNTPs were purchased from Jena Bioscience and the 10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol (DT1) was purchased from SensoPath Technologies (Bozeman, MT). Unless otherwise stated, all reactions were carried out under argon atmosphere with magnetic stirring. The organic solvents were redistilled before use.

Thermosequenase polymerase was purchased from GE Healthcare Life Technology (GE Healthcare Europe GmbH, Spain) and Therminator™ DNA Polymerase from New England BioLabs. Inc, UK. GelRed nucleic acid stain was acquired from Biotium, KAPA2G Robust polymerase from KAPABIOSYSTEMS (distributed by Sigma Aldrich, Spain) and Lambda exonuclease, agarose and DNA Gel Loading Dye (6 ×) from ThermoFisher Scientific, Spain. DNA Clean &Concentrator and Oligo Clean &Concentrator Kits were purchased from Zymo Research, USA.

A model target sequence was designed based on SNPs previously identified within the MYH7 gene present in a group of patients known to suffer from Laing cardiomyopathy (Muelas et al., 2012), where several SNP sites were included in a single PCR amplicon, thus mimicking the SNP sites found in genomic DNA. However, in human genomic DNA these SNP sites are located in different parts of the MYH7 gene and would require multiple amplifications, whereas in our “model” system we have four different SNP sites located in a 103-mer amplicon that is amplified in a single amplification. The sequences were selected using specific oligonucleotide selection and design programs (http://bioinfo.ebc.ee/apex2/). The sequences were designed in such a way to detect the SNPs using four different labels corresponding to the four bases. The HPLC purified oligonucleotides were purchased from Biomers.net, Germany and used as received. The sequences used (from 5`to 3`) are listed below:

Primers and ssDNA template used for PCR amplification:

Forward primer (FW-P)	5′-CGAAGTGTGAACTAGTCCCAC-3′
Phosphorylated Reverse primer (Rv-P)	5′-PO_3-GGGGACTAGGGGACTGAAGAA-3′

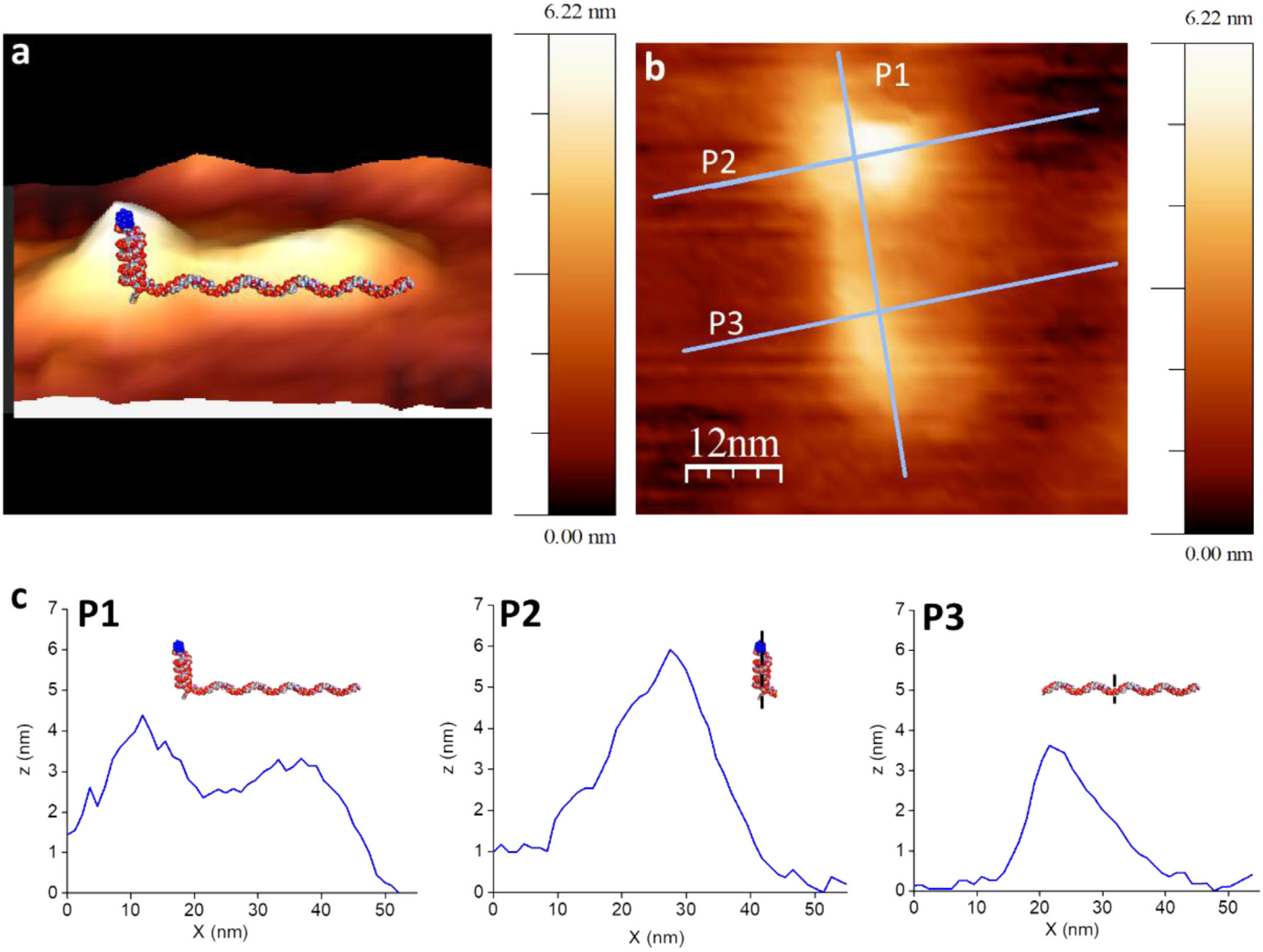

Fig. 2. Topographic AFM images in ambient conditions of the enzymatic incorporation of P_2W_{17}-ddCTP. The 21-mer thiotic acid capture probe was first self-assembled on a 10 nm thick gold coated mica surface followed by hybridisation of a 103-mer ssDNA target before POM incorporation. a) 3D image, b) 2D image including profile lines, c) x-z profiles obtained along the P1, P2 and P3 lines shown in b).

The 103-mer oligonucleotide DNA target/template (a synthetic sequence containing four "SNPs")	5`CGAA***G*** TGTGAACTAGTCCCACCACCTTAA***T*** TTCACTGTGTGTTAACACTTGTAAAG***A*** ACCTGCATAATGTGTGTATCTTAA***C*** TTCTTCAGTCCCCTAGTCCCC -3

The surface-tethered thiolated probes and the short ssDNA sequences containing SNPs used in the electrochemical detection of SNPs are detailed below:

SNP	Surface-tethered thiolated probes:	Short ssDNA sequences containing SNPs:
A	thioctic acid-C5-AGATACACACATTATGCAGGT	AAG***A*** ACCTGCATAATGTGTGTATCT
C	thioctic acid-C5-TACAAGTGTTAACACACAGTGAA	TAA***C*** TTCTTCAGTCCCCTAGTCCCC
G	thioctic acid-C5-AGGTGGTGGGACTAGTTCACA	CGAA***G*** TGTGAACTAGTCCCACCACCT
T	thioctic acid-C5-GGGGACTAGGGGACTGAAGAA	TAA***T*** TTCACTGTGTGTTAACACTTGTA

2.2. Electrochemical measurements

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).

All optimisation studies were carried out with individual 1.6 mm gold electrodes (BAS model MF-2014, 1.6 mm diameter), platinum counter electrode (BAS model MW-1032) and a pseudo-reference electrode of Ag/AgCl (CH Instruments., model CHI111) and the electrochemical measurements were performed in a 1 cm^3 cell. For the multiplexed detection of SNPs, electrode arrays of 36 individual 1 mm^2 square gold working electrodes (150 nm in thick) with a common gold counter electrode and Ag silver reference electrode were used. Fig. SI3). The electrodes were fabricated at Fraunhofer ICT-IMM, Germany using a photolithographic process as previously reported (Fragoso et al., 2011; Joda et al., 2014; Ortiz et al., 2015). (See SI for more details).

Differential Pulse Voltammetry (DPV) was used to detect the incorporation of the labelled ddNTPs. The measurements were performed at room temperature in 10 mM Tris buffer containing 0.5 M NaCl, pH 7. For the three electrodes configuration (optimisation studies), the measurements were recorded in a 1 mL electrochemical cell and for the electrode array (multiplexed studies) in 20 µL of volume. DPVs were recorded at various potential windows depending on the redox

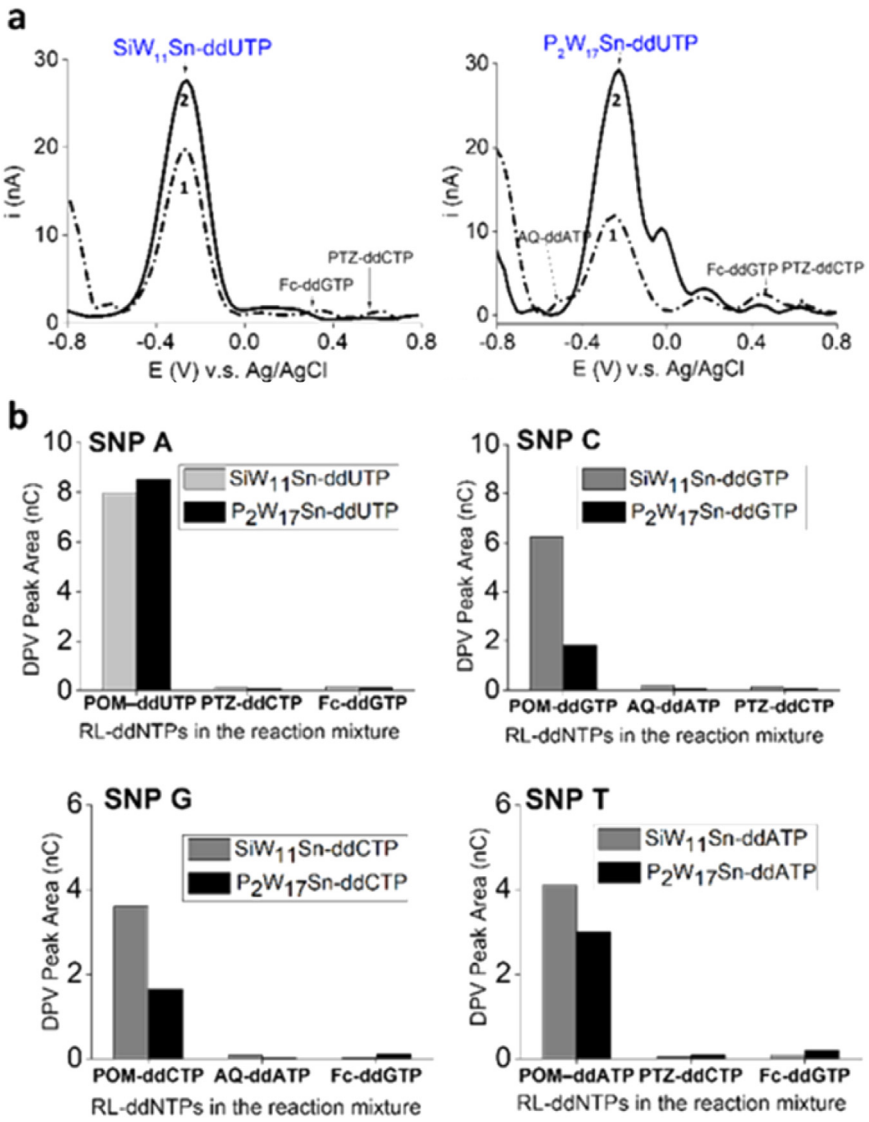

Fig. 3. a) Comparison of the DPV after the incorporation of SiW_{11}-ddUTP (left) and P_2W_{17}Sn-ddUTP (right) at the SNP A position before (1) and after (2) duplex denaturation. b) DPV peak area values of the redox-labelled bases (RL-ddNTPs) present in the reaction mixture for detecting each SNP in a synthetic 103-mer ssDNA. The residual signal coming from the non-specific bases (AQ-ddATP, PTZ-ddCTP and Fc-ddGTP) is also plotted (Average of three electrodes RSD less than 5%).

potential of the labels (vs Ag/AgCl), using a pulse amplitude of 0.1 V, a step potential of 10 mV, a pulse width of 100 ms and a pulse period of 5 ms. For specificity studies and multiplex detection of SNP, the potential window used was from – 0.8 to 0.8 V vs Ag/AgCl, including all the potentials corresponding to the redox labels used in the work reported.

2.3. Atomic force microscopy characterisation

The enzymatic incorporation of P_2W_{17}-ddCTP after 103-mer ssDNA hybridised on thiol surface tethered capture probe on 10 nm thick gold coated mica was studied by atomic force microscopy (AFM). Prior to measurement, the surface was incubated with a 25 mM water solution of $MgCl_2$ to extend the DNA from the surface by minimising the repulsion forces from the gold surface, DNA and polyoxometalate, (Hansma et al., 1993; Herrero-Moreno et al., 2005), and dried with nitrogen. The details of surface preparation and reaction are explained in detail in SI.

The AFM images were recorded in a 5420 at. Force Microscope (AFM) from Agilent Technologies (USA) and processed using WSxM 5.0 Develop 9.2 (Horcas et al., 2007). The surface was scanned in tapping mode at 0.25 ln/min using a SHR-150 kHz frequency tip from NanoAndMore GMBH, Germany. The SHR-150 probe has a single hydrophobic diamond-like carbon extra tip at the apex of a gold coated silicon etched probe. The tip diameter is ca. 1 nm.

3. Results and discussion

Activated polyoxotungstates $[SiW_{11}O_{39}\{Sn(CH_2)_2CO)\}]^{4-}$ (SiW_{11}Sn-Keggin) and $[P_2W_{17}O_{61}\{Sn(CH_2)_2CO)\}]^{6-}$ (P_2W_{17}Sn-Dawson) were coupled with propargyl amino derivatised dideoxynucleotides at position 7 of the purine and 5 of the pyrimidine bases through amide bond formation (Fig. 1) following our established protocol (Boglio et al., 2008; Debela et al., 2014; Debela et al., 2016, Debela et al., 2017) with small variations. A 1.6 mm gold electrode was functionalised with a mixture of thioctic acid ssDNA probe and a bipodal alkanethiol at a ratio of 1:100 (see SI for details).

The optimum buffer for the éPEX reaction and differential pulse voltammetry (DPV) detection was elucidated by evaluating assay performance in Tris-HCl, or phosphate buffered saline (PBS) at pH of 3, 7 and 8. The sharpest and highest DPV peaks was obtained using Tris-HCl at pH 7. Furthermore the use of the two polymerases, Therminator® and

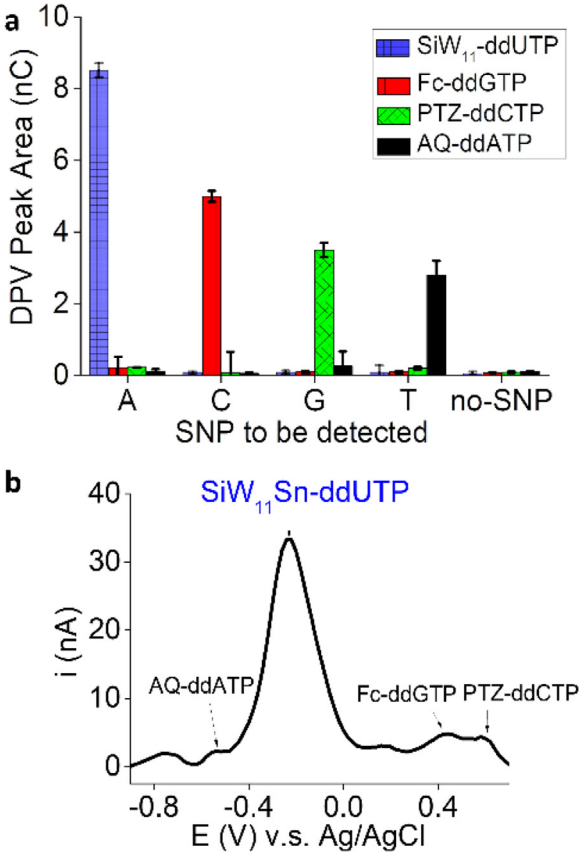

Fig. 4. a) Averaged integrated areas (nC) of the specific and non-specific DPV signals per per SNP interrogated (A for SNP A, C for SNP C, G for SNP G, T for SNP T. Each column represents the value of the DPV signal of each electrochemically active bases (SiW_{11}-ddUTP, PTZ-ddCTP, Fc-ddGTP and AQ-ddATP) measured on the same electrode (Average of six electrodes, RSD less than 5%). b) DPV registered for detecting SNP A by the incorporation of the complementary SiW_{11}Sn-dUTP in 10 mM Tris + 0.5 NaCl, pH 7 (after denaturation at pH 3).

Thermosequenase®, both known to be compatible with modified nucleotide incorporation, were compared. Higher DPV signals were obtained using the Therminator® enzyme, which is also relatively less expensive than the Thermosequenase®. Whilst the optimal temperature for Therminator activity is 75 °C, the use of milder temperatures (24, 42 and 50 °C) were also evaluated. Equivalent performance was observed at 42 and 50 °C, whilst a lower DPV signal intensity was observed at 75 °C, which can be attributed to the thermal cleavage of the thiol-Au bond, resulting in release of the immobilised probe from the electrode surface. No ddNTP incorporation was observed at 24 °C.

Fig. 1b shows the DPV of ddUTPs labelled with SiW_{11}Sn-Keggin and P_2W_{17}Sn-Dawson POMs at SNP A position, using a short (24–mer) synthetic ssDNA target. POMs have different oxidation states as indicated by oxidation peaks at different potentials. Following the éPEX reaction, the main signal is observed at − 0.3 V vs Ag/AgCl for both SiW_{11}Sn-Keggin and P_2W_{17}Sn-Dawson ddNTPs. Once the incorporation of the POM–ddNTP was demonstrated for a short oligonucleotide target sequence, the possibility of carrying out the éPEX reaction using a longer synthetic target sequence was then tested. To demonstrate multiplexing, a 103-mer DNA target containing 4 different "SNP sites" (5`CGA-A***G***T-GTG-AAC-TAG-TCC-CAC-CAC-CTT-AA***T***-TTC-ACT-GTG-TGT-TAA-CAC-TTG-TAA-AG***A***-ACC-TGC-ATA-ATG-TGT-GTA-TCT-TAA-***C***TT-CTT-CAG-TCC-CCT-AGT-CCC-C-3), was amplified using asymmetric PCR followed by exonuclease digestion to generate ssDNA for hybridisation to the surface immobilised probe (Figs. SI1 and 2).

The enzymatic incorporation of POM in the DNA system was visualised by Atomic Force Microscopy (AFM). As observed in Fig. 2, the topographic AFM images obtained using a 1 nm diameter ultrasharp tip show an elongated feature of about 50 nm long and 3–6 nm height, compatible with the size of a 103-mer target hybridised to an immobilised and in situ POM-modified 21-mer probe, in ambient conditions of humidity (Di Santo et al., 2009).

The multiplexed detection of SNPs was carried out using DPV. Following incorporation of the POM-labelled ddNTP, the duplex was chemically denatured using glycine-HCl (pH 3), facilitating increased flexibility and elasticity of the surface tethered POM-DNA, allowing the POM label to move closer to the electrode surface, thus enhancing electron transfer and addressing higher DPV signal (Fig. 3a and SI4). This denaturation step also served to wash away unincorporated labelled ddNTPs and the low pH used can also contribute to the stability of the POM cluster during washing steps.

As can be seen in Fig. 3b, the incorporation of either the Keggin or Dawson labelled ddNTPs e.g. SNP A only shows incorporation of POM-ddUTP and no signal is observed with either AQ-ddATP, PTZ-ddCTP or Fc-ddGTP. The difference in responses for different SNPs can be attributed to different degrees of elasticity of the POM-tethered surface bound DNA, and the dissimilar shapes of the Keggin and Dawson labels. As the signal for SiW_{11}Sn-Keggin was consistently higher than the P_2W_{17}Sn-Dawson for all ddNTPs, they were used as labels for the simultaneous and multiplexed detection of SNPs. Here, an array of 36 electrodes (Fragoso et al., 2011; Joda et al., 2014; Ortiz et al., 2015) was used (Fig. SI3), with each electrode functionalised with a mixture of 1:100 thioctic acid-DNA probe and a bipodal alkanethiol, with six electrodes per SNP, and the remaining twelve electrodes were used as negative controls. A patterned double-sided adhesive gasket was used to create microfluidic channels, and the fluidic housing was completed with PMMA creating a channel with an inlet and outlet for reagent addition (Fig. SI 3). Fig. 4a shows the electrochemical signals for the multiplexed detection of the four SNPs using a mixture of SiW_{11}Sn-dUTP (SNP A), Fc-ddGTP (SNP C), PTZ-ddCTP (SNP G) and AQ-ddATP (SNP T), (Schematic representation shown in Fig. SI 5) and, Fig. 4b shows the highly distinguishable specific signal of SiW_{11}Sn-dUTP (SNP A) from the other non-specific redox labelled ddNTPs.

Moreover, the possibility of also using SiW_{11}-Keggin-ddATP, SiW_{11}-Keggin-ddCTP and SiW_{11}-Keggin-ddGTP for multiplex detection of SNP in combination with other redox labelled bases was also successfully carried out and is reported in Fig. SI6.

4. Conclusions

In summary, we have demonstrated the exploitation of ddNTPs labelled with SiW_{11}Sn-Keggin and P_2W_{17}Sn-Dawson POMs for solid-phase primer extension, and the electrochemical detection of SNPs. The use of the relatively inexpensive Therminator® polymerase was demonstrated to successfully and accurately incorporate the POM-labelled ddNTPs. A post-incorporation denaturation step was observed to increase the DPV signal due to enhanced flexibility and elasticity of the POM-tethered surface bound ssDNA as compared to the rigid duplex. This denaturation step also assisted in the removal of any residual non-specific ddNTPs. SiW_{11}Sn-Keggin ddNTPs gave consistently higher signals as compared to the P_2W_{17}Sn-Dawson ddNTPs and were thus used for the simultaneous and multiplexed detection of SNPs using an electrode array housed within a microfluidic set-up. Four different "SNP sites" were successfully interrogated with negligible background signals. Following this proof-of-concept demonstration, current work is focusing on a higher level of multiplexing for detection of osteoporosis related SNPs (> 20), SNPs associated with rifampicin resistance (7) and SNPs for advanced forensics (> 150).

Acknowledgements

This project has received partial funding from the European Union's

Horizon 2020 research and innovation programme under grant agreement No 767325 and under the Marie Sklodowska-Curie grant agreement No. 659211. We want to thank to Fraunhofer ICT-IMM for provision of gold electrode arrays and the Microscopy and Nanometric Techniques Area of the SRCiT, Universitat Rovira I Virgili, especially to Dr. M. Stefanova Stankova and Dr. L. Vojkuvka for their valuable help in the AFM measurements and surface preparation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2018.06.014.

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