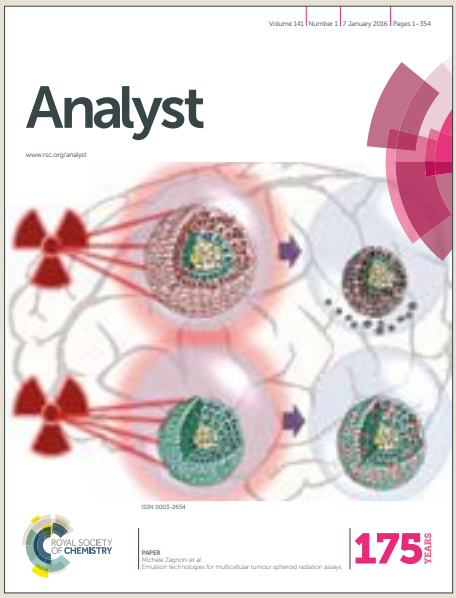
# Analyst

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: S. Petralia, E. L. L. Sciuto, M. L. Di Pietro, M. Zimbone, M. G. Grimaldi and S. Conoci, *Analyst*, 2017, DOI: 10.1039/C7AN00202E.






ROYAL SOCIETY OF CHEMISTRY

rsc.li/analyst

View Article Online
DOI: 10.1039/C7AN00202E

Journal Name

COMMUNICATION

# Innovative Chemical Strategy for PCR-free Genetic Detection of Pathogens by an Integrated Electrochemical Biosensor

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

S. Petralia,[a*] E. L. Sciuto [b], M. L. Di Pietro [c], M.Zimbone [b], M G Grimaldi [b] and S.Conoci [a*]

**An innovative chemical strategy integrated in a miniaturized electrochemical device was developed for sensitive detection of pathogen genome (*HBV virus*) without any amplification step. Results show a Limit of Detection comparable to the standard qRT-PCR method (20 copies/reaction), paving the way to future development of genetic PoC devices addressing automatized and low-cost molecular diagnostics.**

Genetic detection of pathogens is critical for a wide range of applications, including environmental monitoring [1], food control [2], homeland security[3] and, above all, clinical diagnostics[4]. Traditional methods commonly used in the laboratory involve plate counting [5] or detection *via* antibody recognition. They are very laborious, time consuming (48 hours to have a result in case of plate counting) and, often, lack in sensitivity (antibody methods). In the last years, molecular methodologies based on Polymerase Chain Reaction (PCR) [6] have made a real revolution in the diagnosis of infectious microorganisms thanks their ability to combine high specificity and sensitivity with precise quantification and fast response. These advantages make these methods as the prince technologies for molecular diagnostic. However, they are quite complexes needing specialized laboratories and personnel to be execute and rather expensive (20-80$ per test) to be used for massively screening. Therefore, there is a huge demand for methods and systems able to perform analysis of pathogens in sample-in-answer-out format with sensitivity comparable to PCR technologies (LoD 10 copies/reaction) at cost competitive with traditional methods (few $ per test) [7,8]. These are the so-called "genetic Point-of-Care" (PoC), identified by National Institute of Health (NIH) as one of the major priorities for diagnosing of infectious diseases [9]. Genetic PoC will have a relevant world-wide impact. In the developed world they will help the decentralization of diagnostics from the hospital core laboratory to physician office. This would allows massive diagnostic screening and a better facing the threat of pandemics due to the remerging of infectious diseases from globalization [10] and current uncontrolled migration flows. Their clinical utility will be much more relevant in the developing countries where infectious disease diagnosis are still a challenge due to poor clinical laboratory infrastructures and cost constraints [11].

Based on the above considerations, multidisciplinary research teams have spent great efforts over the world to develop miniaturized biosensors based on innovative technologies and new chemical strategies for molecular detection of pathogens. Almost all proposed assays include PCR amplification. In fact, year-to-date PCR is the only biotechnology possible for genetic detection able to guarantee high sensitivity and specificity starting from samples where the DNA target from virus or bacteria is in a very low concentration (LoD < 100 copies/ml of the pathogen). In literature, various PCR-based biosensors have been reported for DNA detection of different pathogen microorganisms. Fernandez-Carballo *et al* in 2016 presented a portable and low cost point-of-care (POC) system based on continuous flow PCR for quantitative detection of Chlamydia trachomatis and Escherichia coli [12]. Hsien *et al* described a sequence-specific electrochemical DNA technology (E-DNA) able to detect up to 100 copies/ml of *S. Typhimurium* by Loop-Mediated isothermal Amplification, 10 TCID50 for H1N1 Influenza virus, 300 copies (in 50ul) *Salmonella Enterica* [13] Other research teams reported miniaturized devices based on PCR using innovative transduction methods for the detection of pathogen species such as Escherichia coli (by piezoelectric-excited cantilever sensors) [14]; Neisseria meningitides (using electrochemical transduction) [15]; Staphylococcus aureus (by means of graphene oxide-based fluorescent probe) [16], Mycobacterium tuberculosis (using Surface Plasmon Resonance) [17], and also for genetic SNPs (Single Nucleotide Polymorphisms) applications [18].

Despite the relevant advantages of PCR-based technologies, their integration in easy-to-use portable devices is still a challenge. In fact, it requires to manage some critical drawbacks mainly related to the (a) biochemistry of the amplification reaction (the presence of enzyme needs surface biocompatibility [19], appropriate storing conditions and absence of inhibitors) and (b) complexity of system architecture that needs accurate thermal control and cycling as well as microfluidic, signal detection and stability of reagent-on-board [20,21].

In this scenario the discovery of PCR-free assay methods suitable to be integrated in miniaturized devices is of fundamental importance in the prospect to develop genetic POC systems. Among the

[a] STMicroelectronics Stradale Primosole, 50 - 95121 Catania (Italy)
[b] Department of Physics and Astronomy University of Catania, viaS.Sofia 64, 95123 Catania, Italy
[c] Dept. Chemical Science University of Messina – Messina (Italy)
*Corresponding authors: salvatore.petralia@st.com and sabrina.conoci@st.com

View Article Online
DOI: 10.1039/C7AN00202E




possible transduction methods for PoC devices, electrical detection is the most preferred since it can be easily interfaced with microelectronic word allowing the integration in smart devices plugged by battery such as mobile phones. In this context, electrochemical detection is the most appealing methodology exhibiting fast response, full compatibility with saline buffer and simple architectures to be designed. Although electrochemical-based DNA sensors exploit a range of several chemical strategies, including redox intercalating molecules [22] or redox-labeled probes [13], the probe immobilization at electrode surface and appropriate surface passivation play a key role in the enhancement of the assay sensitivity and specificity [23].

Motivated by the above considerations, we have developed an innovative chemical strategy able to directly detect pathogen genome without any amplification step with electrochemical signal transduction. As a model, we demonstrate the genetic detection of *Hepatitis B virus* (HBV) from both analytical sample (synthetic clone) and real sample extracted from human blood.

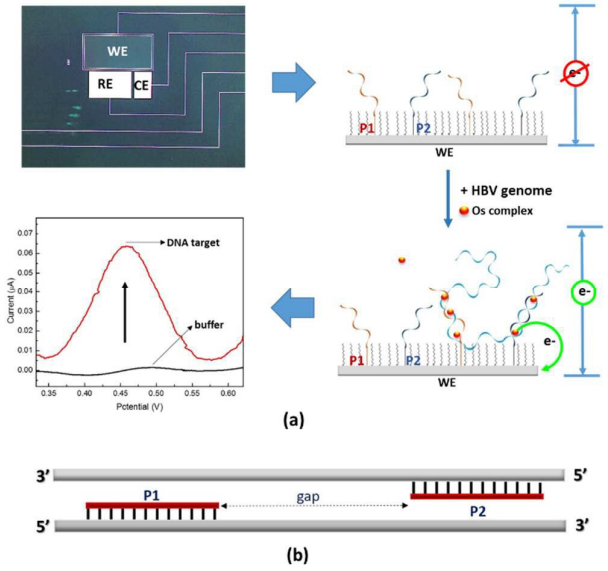



**Figure 1** Operating principle of PCR-free electrochemical pathogen system assay (a) planar biosensor microelectrodes (WE= working electrode, RE= reference electrode, CE= counter electrode) (top-left); WE modified with two specific ss-DNA probes (top-right); HBV genome cooperative hybridization at modified WE with $[Os(bpy)_2DPPZ]Cl_2$ intercalator (bottom-right); observed redox current in presence of hybridised genome (DNA target) or in absence of target (buffer) (bottom-left); (b) P1 and P2 probes design for cooperative hybridization.

Our strategy has been conceived to employ a chemical approach combining (i) a cooperative *in situ* hybridization[24] with two specific probes (P1 and P2) chemically-grafted to a miniaturised Pt-working electrode (WE) surface and (ii) a electrochemical transduction by a redox intercalative Os-complex, [Os(2,20-bipyridine)(dipyrido[3,2-a:2',3'-c] phenazine)]$Cl_2$ ($[Os(bpy)_2DPPZ]Cl_2$) (Fig. 1a). The Os-complex $[Os(bpy)_2DPPZ]Cl_2$ (Supporting Information, Figure S1) has been selected since strongly and specifically intercalates to double stranded DNA with a $K_b$ of 5 x $10^6 M^{-1}$ [22,23]. This complex gives a Square Wave (SW) electrochemical response at about 0.46 V (Supporting Information, Figure S2). P1 and P2 probes were properly designed to recognize specific genetic sequences on both parallel and anti-parallel strands of the same target genome. Moreover, to guarantee the simultaneous hybridization with the two strands of genome target, a sequence gap of 138 bps between the two probes was maintained (Fig 1b) to permit an efficiently genome folding on electrode surface, increasing the hybridization product stability.

This strategy was integrated in a miniaturized silicon electrochemical device composed by an hybridization microchamber (total volume about 20 µl) containing on the bottom surface three planar metal microelectrodes (Pt-Working Electrode (WE) 1000x2000 µm; Au-Reference Electrode (RE) and Au-Counter Electrodes (CE) 800x500 and 800x1250µm; electrode-to-electrode distance 100µm) and resistors for the heating and temperature control during the denaturation and hybridization steps (Supporting Information, Figure S3).

A mixture of two specific thyol 5'-terminated hybridization probes P1 and P2, were immobilized on the Pt working electrode (WE) surface, according to the thiol-Pt chemistry [25]. Then, the WE was passivated by 11-mercaptoundecane to form a self-assembled protective alkyl layer. The success of surface modification via Pt-S-bonds was demonstrated by X-ray photoelectron spectroscopy (XPS) analysis and water contact angle measurements (Supporting Information, Table S1).

The passivation step was found to be fundamental to reduce the electrochemical signal background before target hybridization. SquareWave voltammetry experiments carried out at different $[Os(bpy)_2DPPZ]Cl_2$ concentrations (0.1-10uM) revealed that the best compromise between lowest signal background and highest Os-complex concentration to increase sensitivity was an $[Os(bpy)_2DPPZ]Cl_2$ amount of 5uM (Supporting Information, Figure S4).

Firstly, we demonstrated the validity of the proposed system assay to directly detect pathogen genome, using various amount of synthetic HBV-clone target as analyte, in a concentration range typical for RT-PCR calibrators before amplification (2, 20 200 and 2000 copies/reaction). We chose to start with 2000 copies being a value in the low concentration range of RT-PCR calibrators (typically from $1x10^8$- $1x10^2$ copies/reaction). The experiments were carried out into the miniaturized electrochemical cell above described making firstly a denaturation (at 90°C for 5 min) and then hybridization (30 minutes at 50 °C) in phosphate buffer conditions at pH ~ 7.4 (See Supporting Information). It is has been demonstrated [26, 27] this pH value optimally affects the hybridization. A SW voltammogram was then recorded after hybridization in presence of a solution containing the $[Os(bpy)_2DPPZ]Cl_2$ at concentration of 5 µM. The results gave current density values of 2.6±0.3 µA/cm$^2$ for 2000 copies/reaction, 1.4±0.2 µA/cm$^2$ for 200 copies/reaction and 0.8 ± 0.2 µA/cm$^2$ for 20 copies/reaction, whereas no significant signal was detected for the sample containing 2 copies/reaction (Fig.2a). These data prove a Limit of Detection (LoD) for HBV synthetic genome of 20 copies/reaction. Note that it corresponds to the same order of magnitude for standard real time PCR LoD as illustrated in Figure 2b (right y-axe) (real time PCR details are in SI).

View Article Online
DOI: 10.1039/C7AN00202E




The cross-reactivity of our biosensor system was tested with an unspecific target, the Mycobacterium tuberculosis (MTB) synthetic clone at concentration of ~2000 copies/reaction. The electrochemical results showed that there is no any electrochemical signal when an unspecific target is hybridised on WE modified to HBV probes (Supporting Information, Figure S5).

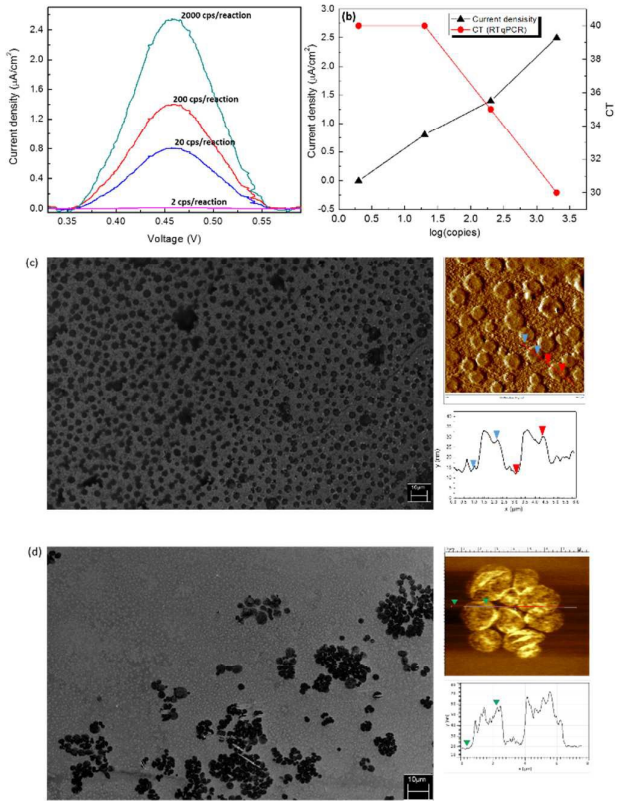



**Figure. 2** – (a) Current density versus Voltage for the testing of HBV-synthetic clone (the hybridization was transduced by 5 μM of $[Os(byp)_2(dppz)]Cl_2$. All measurements performed with scan rate 10mV/sec.); (b) Comparison with standard real time PCR detection; (c) SEM (5.00 kV) and AFM images with cross section profile of hybridized HBV-clone target (2000 copies/reaction) on P1-P2 modified WE electrode; and (d) on unmodified WE surface.

These results prove that our chemical strategy fully reaches the goal to achieve pathogen genetic detection without any amplification step of specific target sequence. We ascribe this outstanding result to an electrical signal amplification due to the presence, at the electrode surface, of long double strand (ds) DNA (a complete genome) captured by two parallel and anti-parallel complementary P1 and P2 probes. In this case, the ds-genome presence considerably increases the amount of the redox $[Os(bpy)_2DPPZ]Cl_2$ intercalating probe very close to the electrode surface producing a strong electrical signal. The WE electrode surfaces inspection after hybridization (2000 copies) with both Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) undoubtedly proves the presence of this ds-HBV genome. Figure 2c,d report the SEM and AFM images for the Pt-WE modified with P1 and P2 and, for comparison, the unmodified Pt-electrode surface. The remarkable finding was the clear presence of HBV-clone structures on modified WE surface, without any sign of aggregation. This can be encouraged by the cooperative hybridization of double strand with the specific P1 and P2 probes. Indeed all clones appear as a single units well separated each other. This is well-supported by the AFM section profile (Fig. 2c) showing a width average value of about 1.5 μm and a height average value ranging from 20 to 40 nm, according to the expected value for a single ds HBV-clone sized 7144 bps.

In contrast, the unmodified WE surface induces the formation of HBV-clone aggregates, to indicate the prevalence of target-to-target interactions, mainly caused by the absences of specific P1 and P2 probes interaction.

Encouraged from these results, we investigated our system assay with real samples, testing an extracted HBV sample from human blood. This sample bearing a starting concentration of $10^7$ cps/reaction (quantified by real time PCR) was diluted to obtain testing solutions containing total DNA amount of 2, 20 200 and 2000 cps/reaction, respectively. Electrochemical experiments were performed according to the previous test with synthetic clone and the results are reported in Figure 3. In this case the positive response is observed up to 200 copies/reaction (LoD) with a current density value of 0.5±0.1 μA/cm$^2$. This LoD value is one order of magnitude lower than that of the analytical sample (synthetic HBV clone) and it should be probably attributed to the presence of interfering species in the extracted sample. Further experiments to gain insights in this point are currently on the way in our laboratories.

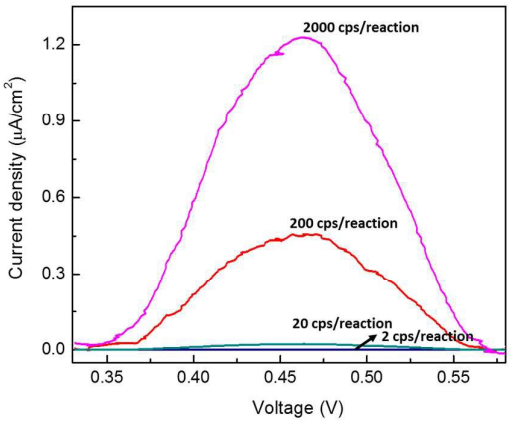



**Figure. 3** - Current density versus Voltage for the testing of HBV extracted sample from human blood (the hybridization was transduced by 5 μM of $[Os(byp)_2(dppz)]Cl_2$. All measurements performed with scan rate 10mV/sec.)

Interestingly, the current density values for analytical samples are always higher than those of the extracted ones at the same HBV genome concentrations (Fig 2a-c). This difference is in excellent agreement with the different chain lengths of the two tested samples. In fact, the analytical sample HBV-clone consists in the genome (3.3kbps) cloned into a plasmid PBR322 vector (3.8 kbps) corresponding to a final circular structure of 7144 bps. On the other hand, the extracted sample does not

View Article Online
DOI: 10.1039/C7AN00202E


include any vector having then the size of HBV genome, corresponding to 3300 bps. This difference results in a higher amount of $[Os(bpy)_2DPPZ]Cl_2$ intercalating into the ds HBV clone, with a consequent higher redox signal.

## Conclusions

In conclusion, to the best of our knowledges, this is the first example reporting a PCR-free assay integrated in a miniaturized electrochemical device, able to detect pathogen genomes without any amplification step with LoD comparable to the standard real-time PCR method. This chemical approach can be easily integrated in portable devices, paving the way to future development of genetic PoC devices addressing automatized and low-cost molecular diagnostics analyses.

## Notes and references

1 J.G.E. Gardeniers and A.Van der Berg, Anal. Bioanal. Chem, **2004**, 378, 17007-1703.
2 I. Palchetti and M. Mascini, Anal. Bioanal. Chem, **2008**, 391, 455-471
3 J.Wang, Anal. Chim Acta, **2004**, 507, 3-10
4 S.O. Kelley, C. A. Mirkin, D. R. Walt, R. F. Ismagilov, M. Toner, E. H. Sargent, Nat. Nanotechnol. **2014**, 9, 969–980.
5 G.H. Weenk, Int J Food Microbiol **1992**, 17, 159-181
6 J.R. Uhl, L.M. Sloan, S.P. Buckwalter, M.F.Jones, E.A.Vetter, J.D.C.Yao, N. L.Wengenack, J. E.Rosenblatt, F. R.Cockerill, and T. F.Smith, Clin. Microbiol Rev. **2006**, 19, 1, 165-256.
7 A. Niemz, T.M. Ferguson, D.S. Boyle, Trends Biotechnol. **2011**, 29, 240-250.
8 S.Petralia, E.L. Sciuto and S.Conoci, Analyst, **2017**, 142, 140-146.
9 D. Mabey, R.W. Peeling, A. Ustianowski, M. D.Perkins, Nat Rev Microbiol **2004**, 2, 231-240.
10 J.H. Nichols. Clin Lab Med **2007**, 27, 893-908.
11 P. Yager, G.J. Domingo, J. Gerdes, Annu Rev Biomed Eng **2008**, 10, 107-144
12 B.L. Fernández-Carballo, I. McGuiness, C. McBeth, M. Kalashnikov, S. Borrós, A. Sharon, A.F. Sauer-Budge, Biomed Microdevices. **2016**, 18, 34-40.
13 K. Hsieh, B.S. Ferguson, M. Eisenstein, K.W. Plaxco and H. Tom Soh, Acc. Chem. Res. **2015**, 48, 911-920 and references therein.
14 K. Rijal, Mutharasan R., Analist 2013, 138, 2943-2950.
15 M.K. Patel, P.R. Solanki, A. Kumar, S. Khare, S. Gupta, Biosens, Bioelectron. **2010**, 25, 2586-2591
16 S. Pang, Y. Gao, Y. Li, S. Liu, X. Su, Analyst **2013**, 138, 2749-2754.
17 H. SH, L. YY, L. SH, T. IF, L. YT, Sensors, **2013**, 14, 458-467.
18 B. Foglieni, A.Brisci, F. San Biagio, P. Di Pietro, S. Petralia, S. Conoci, M. Ferrari, and L. Cremonesi Clin. Chem. Lab. Med., **2010**, 48, 329-336.
19 S. Petralia, G.Ventimiglia Sensors & Transducers Journal, **2012**, 137, 2, 215-225
20 D.R. Almassian, L. M. Cockrell and W.N. Nelson, Chem. Soc. Rev. **2013**, 22, 8769-8798
21 M.O. Spata, M.E. Castagna and S. Conoci, Sensing and Bio-Sensing Research **2015**, 6, 79-84
22 T. Defever, M. Druet, D. Evrard, D. Marchal, B. Limoges, Anal. Chem., **2011**, 83, 1815-1821
23 S. Petralia, M.E. Castagna, E.Cappello, F.Puntoriero, E.Trovato, A. Gagliano, S. Conoci, Sensing and Bio-Sensing Research, **2015**, 6, 690
24 E. Gentalen, M. Chee, Nucl. Acids Res. **1999**, 27, 6, 1485-1491.
25 S.Sortino, S.Petralia, S.Conoci and S. Di Bella, JACS, **2003**, 125, 1122-1123
26 Ulianas, A.; Lee, Y.H.; Sharina, A.H.; Tan Ling Ling., Sensor, **2011**, 12. 5445-5460
27 Ulianas, A.; Lee, Y.H.; Musa, A.; Han-Yih, L.; Zamri, I.; Tan, L.L. Sensor & Actuator B, **2014**, 190. 694-701