Electronic Supplementary Material (ESI) for Analyst.

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

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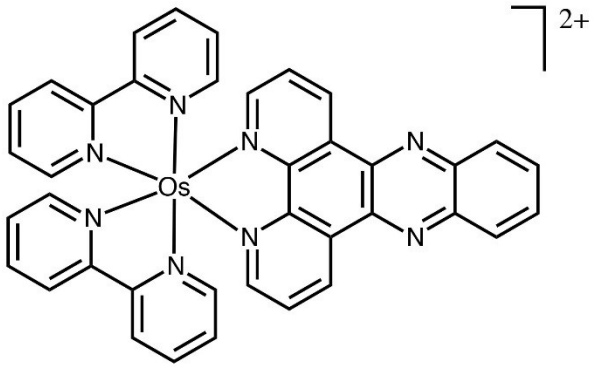

Figure S1 $[Os(bpy)2DPPZ]^{++}$ chemical structure

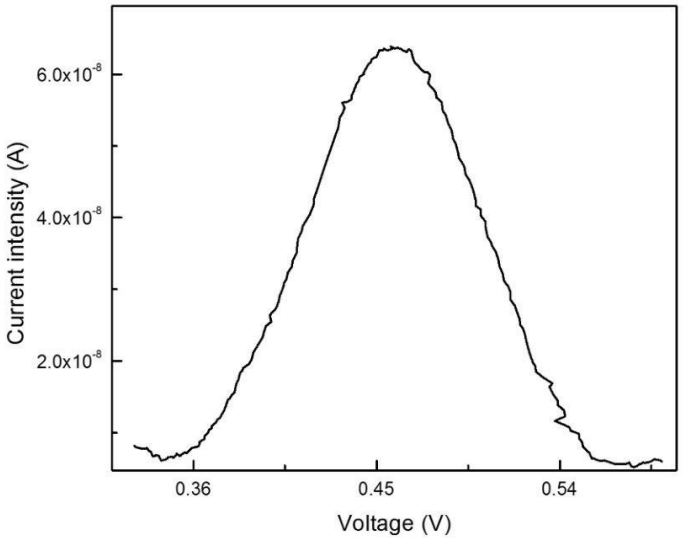

Figure S2 Square Wave (SW) electrochemical response of $[Os(bpy)2DPPZ]^{++}$ at concentration of 10μM.

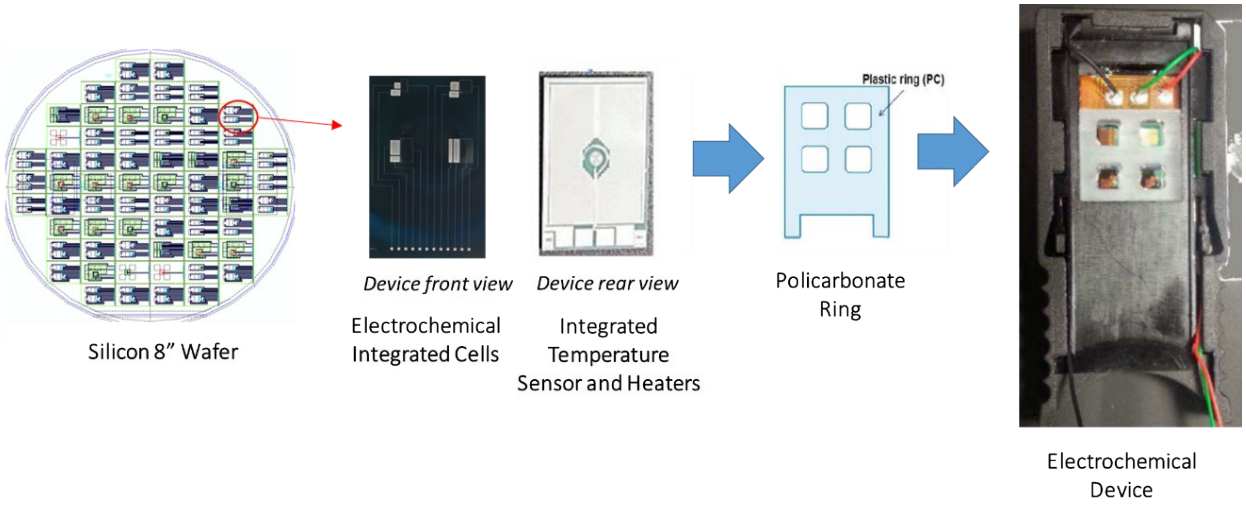

Figure S3 Biosensor main Device fabrication steps.

Table S1. Pt-WE Contact Angle Measurements

Process step	Water Contact angle (°)
Untreated WE surface	121.3 ± 5
CV cleaning	83.3 ± 5
P1 and P2 anchoring	59.6 ± 3
Alkyl passivation layer	88.9 ± 2

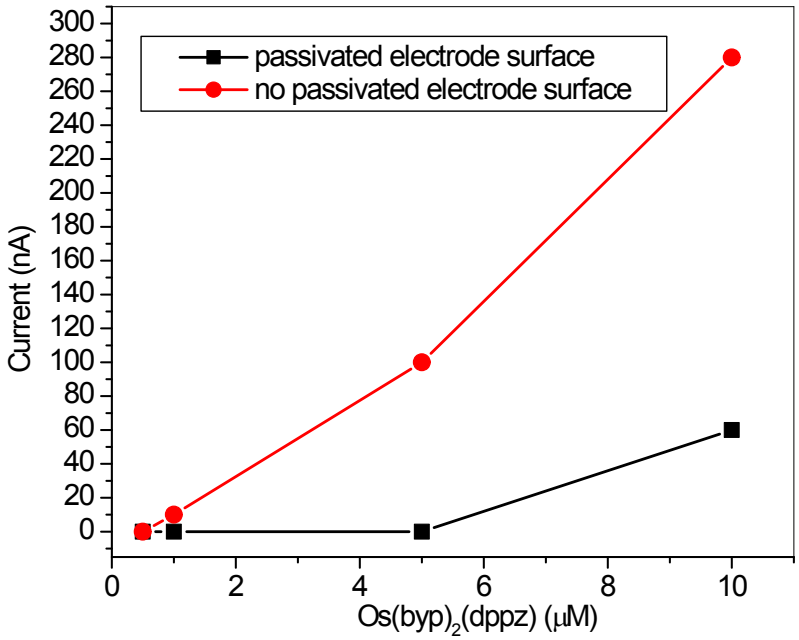

Figure S4. SW measurements on passivated (■) and not passivated (●) modified electrode surface (No HBV hybridization) at different $[Os(bpy)_2DPPZ]Cl_2$ probe concentrations. Not passivated electrode surface (●) exhibited a measurable redox currents over all explored range of the $[Os(bpy)_2DPPZ]Cl_2$ with signal increasing with the increasing of Os-complex concentrations. On the contrary, the passivated surface (■) shows a negligible redox currents up to $[Os(bpy)_2DPPZ]Cl_2$ concentration of 5uM. This data demonstrates that alkyl-thiol layer guarantee an effectiveness surface passivation providing a diffusion barrier for unspecific redox signal

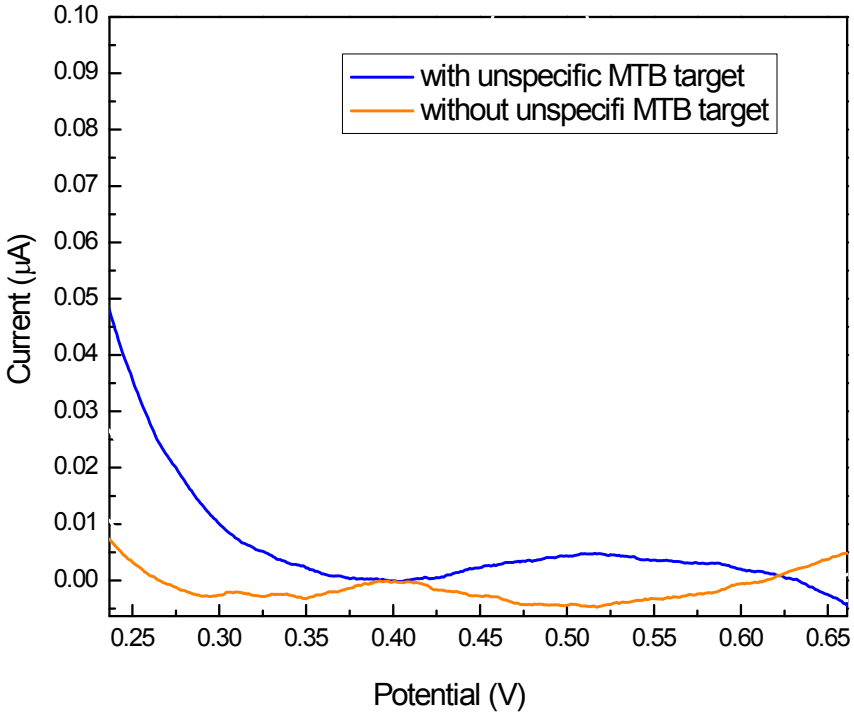

Figure S5. SW voltammograms for hybridization with unspecific DNA target (MTB-clone ~2000 cps) with an amount of $[Os(bpy)_2DPPZ]Cl_2$ redox probe of 5 uM (blue line) and without any target with an amount of $[Os(bpy)_2DPPZ]Cl_2$ redox probe of 5 uM (orange line) .

Experimental Procedures

Chemicals

P1 probe (sequence: 5' HS-C6-GGT GAG TGA TTG GAG GTT) and P2 probe (sequence: 5'HS-C6-CAC ATC AGG ATT CCT AGG) were purchased from MWG (Germany).

Hepatitis B virus (HBV) clone complete genome was purchased from Clonit (ref. 05960467) and consists in HBV genome 3.2 kbps and a plasmid PBR322 vector 3.8 kbps provided in a TE (Tris 10 mM,EDTA 1 mM, pH = 8) solution.

Micobacterium Tubercolosis (MTB) clone complete genome was purchased from Clonit (ref. 05960564) provided in a TE (Tris 10 mM,EDTA 1 mM, pH = 8) solution.

Qiagen QIAamp DNA Mini Kit (Ref. 51306) was purchaised by Qiagen

KCl powder and 11-mercaptoundecane were purchased from Sigma Adrich.

All reagents used for the synthesis of $[Os(bpy)_2DPPZ]Cl_2$ (see below) were puchaised by Sigma Aldrich.

Synthesis and Characterization of [Os(2,2'-bipyridine)(dipyrido[3,2-a:2',3'-c]phenazine)]Cl2 ([Os(bpy)2DPPZ]Cl2)

The compound $[Os(bpy)_2DPPZ]Cl_2$ was prepared by coordination of dipyrido[3,2-a:2',3'-c]phenazine (DPPZ) to $[Os(bpy)_2Cl_2]$ according to published procedures [1]. The DPPZ was prepared according to published methods [2] with some modifications. The compound $[Os(bpy)_2DPPZ]Cl_2$ was prepared as follows: 30 mg of $[Os(bpy)_2Cl_2]$ were dissolved in 10 ml of ethylene glycol, together with 1.2 molar equivalents of DPPZ. The solution was maintained under continuous magnetic stirring and refluxed under nitrogen atmosphere. After 6h the solution was cooled to room temperature. An excess of solid NH_4PF_6 and 10 mL of diethyl ether were added to the mixture. By adding few drops of methanol for combining the phases a dark brown precipitate was obtained. The crude product was filtered off, washed with water and diethyl ether. The solid was purified by column chromatography on neutral alumina with acetonitrile-toluene (3:1, v:v) as eluent. The first brown-green fraction was evaporated under reduced pressure. 5 ml of acetone was used to dissolve the solid. By adding an excess of ter-butyl ammonium chloride a dark brown solid was obtained as chloride salt (28 mg). The compound was fully characterized by 1H-NMR (CD3CN): 9.40 (d, 2H), 8.45 (dd, 2H), 8.40 (dd,4H), 8.30 (s, 4H), 8.18 (dd, 2H), 8.12 (dd, 2H), 8.05 (d, 2H), 7.90 (d, 2H), 7.70 (dd, 2H), 7.60 (dd, 2H), 7.58 (dd, 2H).

The Elemental Analysis founded was C, 42.40; H, 2.34; N, 10.22 was in good agreement with the calculation data for a compound $C_{38}H_{26}F_{12}N_8OsP2$: C, 42.46; H, 2.44; N, 10.43.

Figure S1 report the molecular structure of $[Os(bpy)_2DPPZ]^{++}$, according to literature [3] the complex can reversibly exchange one-electron at a standard potential (E° ranging from 0.1 to 0.8 V vs SCE).

Silicon Electrochemical Device Fabrication

The silicon electrochemical device has been manufactured using the VLSI technology on 8" silicon substrate, as described in our previous work [4]. The biosensor is manly composed by three layer: the first layer is a silicon resistor, for the heating and temperature control, a silicon wafer containing the electrochemical cell (based on three planar microelectrode) is the second layer bonded to the first and finally a polycarbonate ring mounted upon the second layer to form the four microchambers. A black plastic holder guarantee the easily device handling (Figure S3).

To electrically isolate the electrodes from the substrate a first silicon oxide layer has been thermally grown. Then a platinum film has been sputtered and lithographically defined in the electrodes areas and contact zones (PAD). A second passivation layer was then deposited by PECVD tecniques to isolate the Pt layer. A dry etch was performed to connect the first and the second metallization. The second metallization (Au) was then sputtered and lithographically defined in complementary electrodes regions and contacts areas. Each electrochemical cell is composed by three planar electrodes: a working electrode in platinum with size 1000 × 2000 μm, a counter and a reference electrode made in gold with size 800 × 500 and 800 × 1250 μm, respectively. The electrode-to-electrode distance are 100 μm.

Pt- WE Surface Chemical Modification

The Platinum working electrodes (WE), were properly chemical modified as follows: the WE was firstly cleaned by 10 CV cycles from -1 to 1V in Potassium Chloride 5 mM buffer. After that 10 μl of a solution containing sodium phosphate buffer (150 mM at pH9.0) and the two specific thyol 5'-terminated probe (P1 and P2) at final concentration of 20 μM was microdeposed (Perking Elmer Piezo array) on the WE surface and incubated in a climatic chamber at 30 °C, 90% RH for 4 hours. After several cleaning with deionized water to remove the unbinding P1 and P2 probes, the WE-modified surface was passivated (by microdeposition) with an ethanolic solution of 11-mercaptoundecane (10 mM). Then, several ethanol washing followed by three washing in water of the electrode were carried out. All reactions were performed in a 1000 clean room.

Pt- WE Surface Characterizion

a) X-ray photoelectron spectroscopy (XPS) analysis.

XPS spectra were obtained an AXIS-HS spectrometer by Kratos Analytical. The Mgkα radiation of 1253.6 eV at the condition of 10 mA and 15 keV have been used. The energy analyzer was set to a pass energy of 40 eV. The binding energy scale was referred to the C 1s component centered at the lowest binding energy value, assumed at 285 eV. XPS analysis provided clear evidence that the electrode chemical modification (P1 and P2 grafting and alkyl passivation step) occurred efficiently. The finding data shown a broad peak for C1s, it can be deconvoluted in three main components, the first centered at 285 eV of

binding energy is assigned to C-C, C-H chemical bonds; the second component centered at 286.7 eV is assigned to the carbon atoms in the phosphodiester structure -C*H2-O-PO2- characteristic of the polynucleotide chain [5] and the last peak centered at 288.3 eV assigned mainly to the carbon atoms bonded to two vicinal nitrogen atoms N-(C*H)=N [6] for the of ring bases. In addition, the N 1s peak centered at about 400 eV of binding energy was assigned to the nitrogen atoms of nucleic acids base. Finally the signal at 162 eV was diagnostic for the S element present on both P1 and P2 probe and 11-mercaptoundecane passivation molecule.

b) Contact angle measurements

Contact angle measurements were carried out on test chip consisting in 1x1cm Pt surface after each chemical modification step using a Theta-meter. Three drops of distilled water of 1µl each were dispensed on the surface of the sample. For each drop two contact angle values (the right and left angles) were taken, for a total of six measurements for each sample. Table S1 show the CA values that highlighted clear chemical surface modification.

Square Wave (SW) Measurements.

SquareWave voltammetry (SW) measurements were recorded by a Parstat 2273 (Princenton Applied Research) equipment interfacing the miniaturized silicon electrochemical cell with the follow conditions: Square-Wave (SW), scan rate 10mV/s, pulse high/pulse width 0.025V for 0.05s, stop height 12mV. Electrochemical measurement were carried out in the device microchamber (volume 20µl) using Potassium Chloride 5 mM as buffer with the follow conditions: scan rate of 10mV/s, pulse high/pulse width 0.025V for 0.05s and stop height 12mV.

SEM and AFM Instrumentation.

AFM analyses were performed with a Veeco-Innova microscope operating in amplitude mode, and ultra sharpened Si tips were used (MSNL-10 from Veeco Instruments, with anisotropic geometry, radius of curvature ~2 nm, tip height ~2.5 µm, front angle ~15°, back angle ~25°, side angle 22.5°). AFM images were analyzed by Gwyddion free software.

SEM images were obtained by using high performance Schottky field emission LEO 1550 SEM. Instrument operating at 5 kV in secondary electron imaging mode.

Hybridization Experiments (HBV genome)

The HBV genome hybridization on Pt-WE modified surface with P1 and P2 probe was carried out for 30 minutes at 50 °C in 20 µL of total volume containing various amount of the DNA target HBV clone (2000,

200, 20 and 2 copies) in 20mM sodium phosphate buffer, 1M NaCl, 5.2mM KCl, 0.1% Tween 20, 2x Denhardt's solution. After hybridization the electrode was washed three times with deionized water.

Cross-reactivity with Mycobacterium tuberculosis (MTB)

The cross-reactivity of our system assay was investigated by testing the Pt-WE modified with P1 and p2 probes specific for HBV-clone with unspecific target consisting in Mycobacterium tuberculosis clone (MTB complete genome purchased from Clonit (ref. 05960564)). The hybridization was carried out according the procedure described in the "Hybridization Experiments (HBV genome)" section. The electrochemical results (Figure S5) shows negligible SW signal for the hybridization carried out with a total amount of 2000 copies of MTB-clone (blu line). For comparison SW signal before hybridization is also reported (orange line).

Real Time PCR Experiments.

Real Time PCR experiments were executed on standard 96 well reaction plate using Applied Biosystem 7500 real time PCR equipment. Experiments were performed using a Clonit kit ref product CLO-FO2 HBV MMIX KIT 48 and used according to the Instruction for Use. 10 µL of a Master Mix solution (purchased by Clonit) containing PCR buffer (1×), Taq DNA polymerase, 0.5 µM of each forward and reverse primers and 2 µL of standard HBVclone (2, 20 200 and 2000 total copies). The PCR cycles were: denaturation step 10 min. at 95°C, followed by 45 cycles of 95°C for 15s and 60°C for 60s. Negative samples were prepared substituting the HBV clone with the same amount of water. Each experiment was replicated 3 times.

Real Sample DNA extraction.

HBV genome extraction from human blood was carried out using the commercial kit Qiagen QIAamp DNA Mini Kit (Ref. 51306), according their Instructions for Use. All experiments were performed in compliance with the Approved Guideline *MM13-A: Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods*, by Clinical and Laboratory Standards Institute (CLSI), recognized as consensus guideline by the U.S. Food and Drug Administration (FDA). The extraction experiments we executed in laboratories certified under **ISO 9001** and **ISO 13485.**

Supplementary References

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