A miniaturized silicon based device for nucleic acids electrochemical detection

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ABSTRACT

In this paper we describe a novel portable system for nucleic acids electrochemical detection. The core of the system is a miniaturized silicon chip composed by planar microelectrodes. The chip is embedded on PCB board for the electrical driving and reading. The counter, reference and work microelectrodes are manufactured using the VLSI technology, the material is gold for reference and counter electrodes and platinum for working electrode. The device contains also a resistor to control and measuring the temperature for PCR thermal cycling. The reaction chamber has a total volume of 20 μL. It is made in hybrid silicon–plastic technology. Each device contains four independent electrochemical cells.

Results show HBV Hepatitis-B virus detection using an unspecific DNA intercalating redox probe based on metal–organic compounds. The recognition event is sensitively detected by square wave voltammetry monitoring the redox signals of the intercalator that strongly binds to the double-stranded DNA. Two approaches were here evaluated: (a) intercalation of electrochemical unspecific probe on ds-DNA on homogeneous solution (homogeneous phase); (b) grafting of DNA probes on electrode surface (solid phase).

The system and the method here reported offer better advantages in term of analytical performances compared to the standard commercial optical-based real-time PCR systems, with the additional incomes of being potentially cheaper and easier to integrate in a miniaturized device.

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1. Introduction

Nucleic acid analysis has been an attracting topic in fields such as gene analysis, pathogen detection, environmental and forensic analysis [1–3]. In this scenario polymerase chain reaction (PCR) has become a common method for nucleic acids detection. In this field, scientists had developed a series of technologies, such as multiplex PCR, isothermal PCR, real time PCR and reverse transcriptase PCR. Recently the scientific community has focused its attention on the development of miniaturized microfluidic chips, made of silicon or plastic material, suitable to perform PCR on a small sample volume (<25 μL) [4]. The main advantages of these chips include low the cost of analysis due to the low volume of reagent and sample, the low response time and the ability to integrate upstream and downstream process such as sample preparation and detection directly on chip [5]. Several microfluidics chips are describes in the literature performing nucleic acids amplification.

However, only few examples that include nucleic acids detection by real time PCR are reported [6] and the most of these are based on optical detection methods.

The immobilization of oligonucleotides onto surfaces has been reported as one of the most successful strategy to enhance the sensitivity for biosensor systems [7–9]. In 2008, Hsing demonstrated, for the first time, the possibility to electrochemically monitor DNA during the amplification process on a solid phase PCR, through the incorporation of redox-labeled base during the amplification [10,11]. Limoges et al. proposed a novel electrochemical detection method that indirectly detects DNA polymerization in homogeneous phase [12]. In particular, PCR process is detected in real time by monitoring the electrochemical signal of a intercalating redox probe, based on osmium complexes [13], that remains free in solution in presence of amplified DNA: the final result is an exponentially decreasing of the signal of the redox intercalation with the increasing of the amplified PCR sample by the PCR cycles.

Intercalating molecules such as metal complexes based on ruthenium, osmium, iridium, platinum, cobalt [14–17] or organic compounds [18] may be a good method for electrochemical DNA probing because of their advantages such as reversibility of the redox reaction, chemical

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stability and simple functionalization. Recently researcher reported the use of bypyridine (bpy) and dipyridophenazine (DPPZ) osmium (II) as luminescence and electrochemical probes for real-time method. However it is difficult to employ it as electrochemical probe since its high redox potential can destroy the species immobilized on the electrode (guanine and adenine oxidation).

In this paper we reported a novel silicon based device for nucleic acids detection based on the electrochemical monitoring of a unspecific DNA intercalating probe based on osmium complex. The miniaturized silicon chip integrates planar microelectrodes together with temperature sensors and heaters manufactured by using the standard VLSI technology. A PCR chamber is defined by a polycarbonate structure, so that a total reaction volume of 20 μL is achieved. The chip is embedded on PCB board for the electrical driving and reading.

To demonstrate the ability to electrochemically detect DNA, experiments using HBV (Hepatitis B virus) clone as target and $Os[(bpy)_2DPPZ]^{2+}$ as probe were performed. Two approaches were here evaluated: (a) intercalation of electrochemical unspecific probe on ds-DNA on homogeneous solution (homogeneous phase); (b) grafting of DNA probes on electrode surface (solid phase). In the first approach the detection is proven by the decrease of redox signal due to the less easily electrochemically detectable probe intercalated into ds-DNA, while in the second one the DNA detection is confirmed by the increasing of redox signal with the increasing of PCR cycles.

2. Materials and methods

2.1. Chemicals

The osmium complex [Os(2,2′-bipyridine)(dipyrido[3,2-a:2′,3′-c]phenazine)]Cl$_2$ (Os(bpy)$_2$(DPPZ)$^{++}$) was synthesized according to published procedures [17]. According to literature [12], the complex can reversibly exchange one-electron at a standard potential ($E°$ ranging from 0.1 to 0.8 V vs SCE).

Hepatitis B virus (HBV) clone (ref. product CLO-05960116 HBV Complete Genome) an all the reagents for the HBV real time PCR were purchased from Clonit (kit ref. product CLO-FO2 HBV MMIX KIT 48) and used according to the *Instruction for Use*.

Human Genomic DNA (10 ng/μL) was purchased from Jena Bioscience.

Thiolated HBV capture probe 25-mer long was supplied form Clonit.

2.2. Amperometric device

The silicon electrochemical device has been manufactured using the VLSI technology on a 6″ silicon substrate. To electrically isolate the electrodes from the substrate a silicon oxide layer has been firstly thermally grown (first passivation layer). 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 (Silicon oxide by PECVD) to isolate the first metallization (Pt) form the second one (Au). A dry etch was performed to connect the first and the second metallization (i.e., on interconnection tracks). The second metallization (Au) was then sputtered and lithographically defined in complementary electrodes regions and contacts areas. Fig. 1 shows a schematic cross section of the electrochemical cell structure.

The chip is then assembled with a second silicon device containing integrated temperature sensors and heaters (Fig. 2a). The final silicon device is mounted on a polycarbonate ring to create 4 reaction chambers of 20 μL each that contains on their floor the electrochemical electrodes. The complete structure is fixed on a plastic holder for easily handling (Fig. 2c).

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 distances are 100 μm and 200 μm. Fig. 2b reports a scheme of the electrochemical cell layout.

2.3. Homogenous phase experiment (real time PCR)

The real time electrochemical PCR experiments were performed using a master mix solution of 20 μL containing Clonit buffer (1×) and Hot start polymerase, 0.5 μM of each forward and reverse primers, 2 μL of HBV-clone (10^5 copies/μL). Different amount of Os(bpy)$_2$(DPPZ)$^{++}$ ranging from 0.1 to 1 μM were added. The PCR cycling was performed in a portable thermalcycler (Q3-thermocycler developed by STMicroelectronic – Fig. 2d) by using the following thermal program: preheating period of 10 min at 95 °C, followed by a maximum of 45 cycles of 95 °C for 15 s and 60 °C for 60 s.

The square wave voltammograms were recorded at the end of the PCR cycles.

Same real time PCR experiments (20 μL of the above reported master mix) were executed on standard tube in Applied Biosystem 7500 real time PCR equipment.

2.4. Solid phase experiment (hybridization on immobilised capture probe)

The working electrode of the electrochemical device has been functionalized by spotting of a solution containing 10 μM of thiolated HBV capture probe (25-mer long). The chip was incubated for 4 h at 30 °C (90% RH) and washed by deionized water and dried by nitrogen flow.

The functionalized working electrode was hybridized (60 min at 55 °C) with solutions (20 μL) of HBV PCR-cycled at 0, 20 and 40 PCR cycles, respectively. After hybridization, a solution (2 μL) containing 0.1 μM of redox probe was added and square wave curves recorded.

2.5. Electrochemical measurement

The square wave voltammetry measurements were recorded by a Parstat 2273 (Princenton Applied Research) equipment with the follow conditions: square-wave (SW), scan rate 10 mV/s, pulse high/pulse
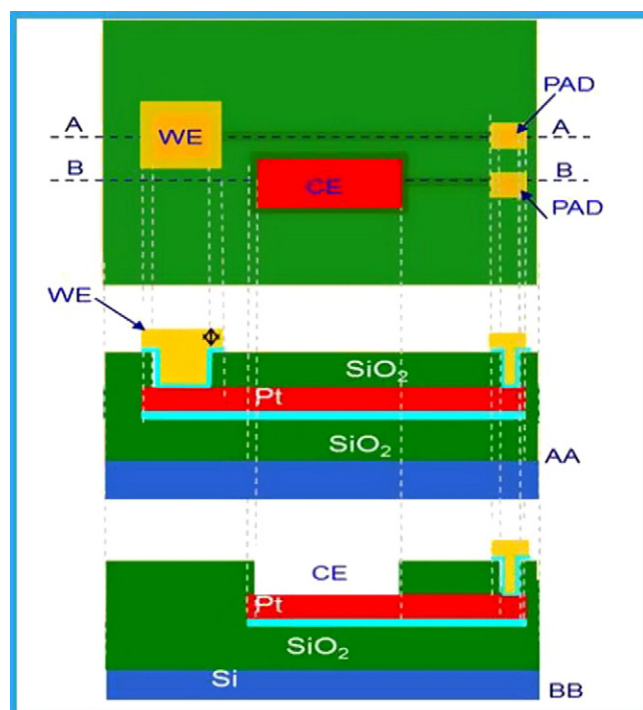

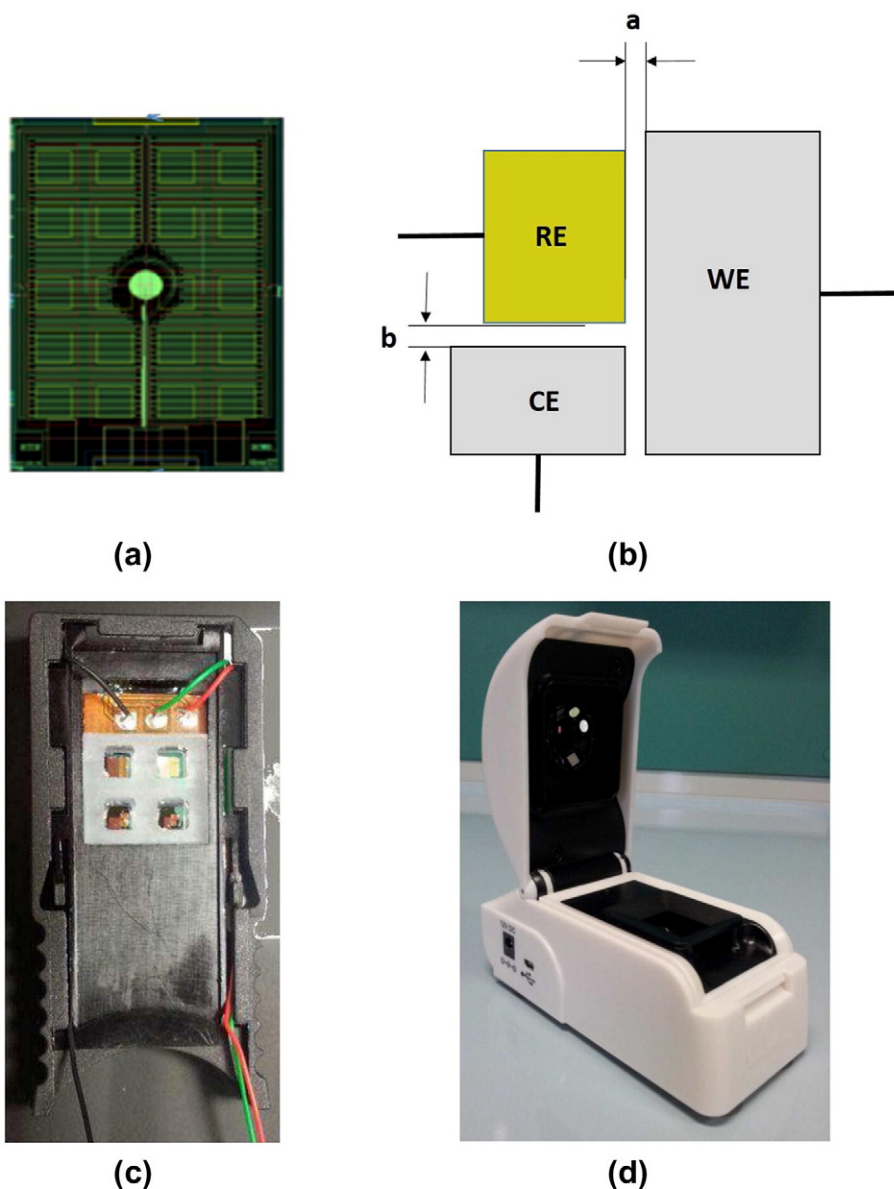**Fig. 1.** Schematic cross section of the electrochemical cell.

Fig. 2. (a) Temperature sensors and heaters, (b) electrochemical cell, (c) amperometric device, and (d) portable Q3-thermalcycler.

width 0.025 V for 0.05 s, stop height 12 mV. All electrochemical measurement were carried out in potassium chloride 20 mM buffer.

For the solid state intercalation test a sodium phosphate 150 mM solution of oligonucleotide 5′-thiol terminated was grafted at working electrode surface overnight at room temperature, washed several time with water and dried by nitrogen flow.

3. Results and discussion

To evaluate possible PCR inhibition effect by osmium complex, real time PCR experiments in standard microtubes were performed. In particular, different redox probe concentrations ranging from 0.1 to 8 μM were tested. The results are reported in Fig. 3. It can be noticed that no PCR inhibition occurs at osmium complex concentrations in the 0.1–1 μM range, while at upper concentration values the PCR was quite inhibited. The C_T values measured at the osmium probe concentrations of 0.1, 0.5 and 1 μM were 21.35, 21.98 and 22.00 respectively. According to no inhibition effect, these values are comparable with what obtained with a master mix without Os complex (C_T equal to 21.94).

The compatibility of PCR with the electrochemical cells was investigated by performing real time PCR experiments into the electrochemical device. For this purpose, 10 μL of HBV mix (10^5 copies/μL) were cycled in the each of the four reaction chambers of the electrochemical device and read in real time by the Q3 reader (FAM labeled probe). The mean value of C_T obtained in the 4 chambers was 22.9 ± 1.2, quite comparable with the number obtained in the microtubes (C_T 23.9 – see above). These data clearly indicate that the device structure and the materials that compose the main modules of PCR chamber (gold and platinum for planar electrodes, silicon oxide and polycarbonate of chamber walls), did not inhibit the DNA amplification reaction.

In order to verify the capability of the system to detect redox probe signals upon unspecific intercalation, square wave measurements were recorded directly on the electrochemical cell by using different human genomic DNA amounts. Fig. 4 reports the decrease of redox signal for a solution 0.125 μM of Os(bpy)$_2$(DPPZ)$^{++}$ in potassium chloride buffer 20 mM in absence and in presence of 50 and 100 ng of human DNA, respectively. The decrease of signal clearly indicates the effectiveness probe intercalation. It is completely intercalated with 100 ng of *human* DNA.
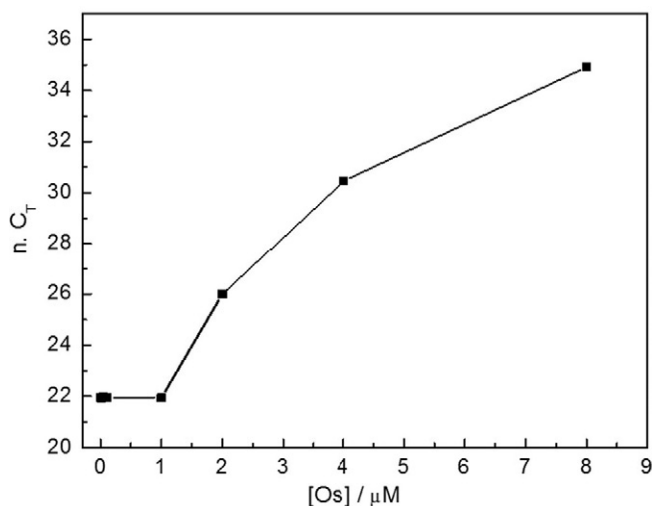
Fig. 3. PCR inhibition effect at different Os probe concentration.

Fig. 5. Square wave curves for redox probe 0.1 µM at different PCR cycles.

To evaluate the ability of the system to detect amplified product in homogeneous phase, square wave signals were recorded on solutions (20 µL) containing a redox probe concentration of 0.1 µM and PCR product amplified at different cycles (0, 10, 20, 30 and 40 cycles). Data are showed in Fig. 5. It can be noticed that the electrochemical response agrees with the intercalation process since the electrical signal decreases with the increasing of the DNA amounts (inserted figure).

The affinity binding of $Os(bpy)_2(DPPZ)^{++}$ with PCR product was measured by the Scatchard plot (data not showed) [19]. A K_b value of $1.5 \times 10^6 \, M^{-1}$ was found. This is in agreement with both the $K_b = 4 \times 10^6 \, M^{-1}$ determined for calf thymus DNA and $K_b = 5 \times 10^6 \, M^{-1}$ determined for PCR product [12].

To finally demonstrate the efficiency to detect the PCR amplification in solid state, a thiolated HBV capture probe 25-mer long was grafted on working electrode surface. The layer was hybridized with HBV PCR-cycled product in presence of 0.1 µM of redox probe. Fig. 6 reports the square wave curves (SW) recorded (solid line). For comparison the same curve (PCR-cycled product containing 0.1 µM of redox probe) obtained by measurements in solution (dashed line) is reported. The SW curve at solid phase shown a significantly shift of peak voltage at higher values (from 0.66 to 0.81 V) together with a huge increasing of sensitivity. In addition, the peak current increases from 1.8×10^{-6} A for the intercalation on homogeneous phase to 5.6×10^{-6} A in case of the solid
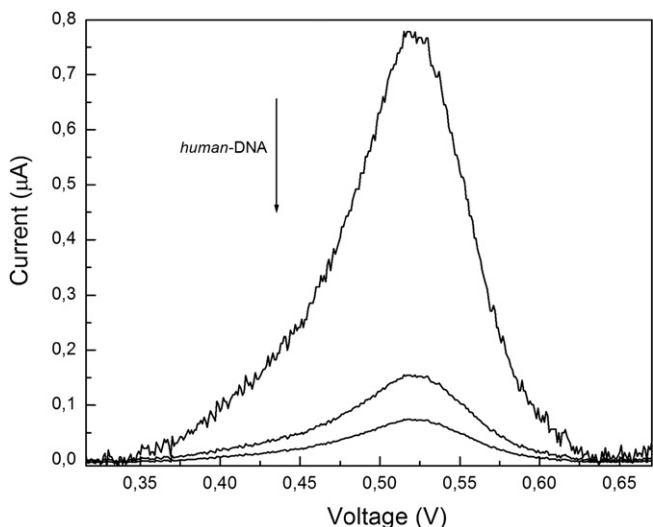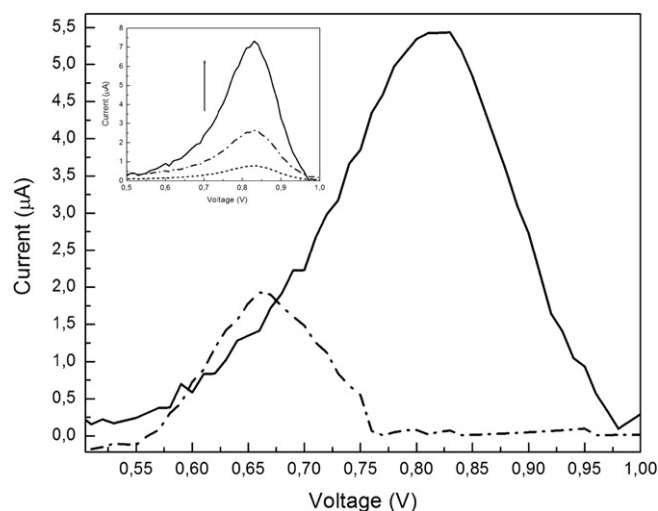

Fig. 6. Square wave curves of redox probe for solid state (solid line) and homogenous phase (dash line) PCR products intercalation. Inserted the redox signal increasing for 0, 20 and 40 PCR cycles.

state. This is in agreement with literature data for the redox process with monolayer immobilized on electrode surfaces.

4. Conclusion

In this paper we have described a novel portable system for nucleic acids electrochemical detection. Two different approaches were investigated: (a) intercalation of electrochemical unspecific probe on ds-DNA on homogeneous solution (homogeneous phase); (b) grafting of DNA probes on electrode surface (solid phase). The data here presented confirms the ability of electrochemical cells to detect the PCR amplified products with both the approaches. Real time PCR data, recorded in homogenous phase, prove the compatibility of the PCR process towards both the presence of redox probe $Os(bpy)_2(DPPZ)^{++}$ (concentration range 0.1–1 µM) and the electrochemical cell materials. However, the solid phase approach reveals a better potentiality for future applications. Actually, it is noteworthy that an increasing of sensitivity of about three times was found due

Fig. 4. SW curves for redox probe 0.1 µM at different human DNA amount.

to the intercalation on ds-DNA directly grafted to the electrode surface.

The miniaturized system here proposed could be a good candidate for point-of-care applications in the medical field.

Acknowledgments

References

[1] E. Palecek, M. Bartosik, Electrochemistry of nucleic acids, Chem. Rev. 112 (2012) 3427–3481.

[2] C.-H. Lu, B. Willner, I. Willner, DNA nanotechnology: from sensing and DNA machines to drug delivery systems, ACS Nano 7 (2013) 8320–8332.

[3] A. Merkoci, A. De la Escosura-Munniz, M. Espinoza-castaneda, Nanoparticles/nanochannels – based electrochemical biosensors, J. Nanosci. Nanotechnol. 96 (2015) 205–223.

[4] B. Foglieni, A. Brisci, F. San Biagio, P. Di Pietro, S. Petralia, S. Conoci, M. Ferrari, L. Cremonesi, Integrated PCR amplification and detection processes on a Lab-on-Chip platform: a new advanced solution for molecular diagnostics, Clin. Chem. Lab. Med.: CCLM 48 (3) (2010) 329–336.

[5] S. Petralia, R. Verardo, E. Klaric, S. Cavallaro, E. Alessi, C. Schneider, In-Check system: a highly integrated silicon Lab-on-Chip for sample preparation, PCR amplification and microarray detection of nucleic acids directly from biological samples, Sens. Actuators B: Chem. 187 (2013) 99–105.

[6] M.S. Ibrahim, R.S. Lofts, P.B. Jahrling, E.A. Henchal, V.W. Weedn, M.A. Northrup, P. Belgrader, Real-time microchip PCR for detecting single-base differences in viral and human DNA, Anal. Chem. 70 (9) (1998) 2013–2017.

[7] A. Erdem, Nanomaterial based electrochemical DNA sensing strategies, Talanta 74 (2007) 318–325.

[8] G. Ventimiglia, S. Petralia, Recent advances in DNA microarray technology: an overview on production strategies, detection methods and applications, Bionanoscience 3 (2013) 428–450.

[9] M.M. Rahman, X.B.B. Li, N.S. Lopa, S.J. Ahn, J.J. Lee, Electrochemical DNA hybridization sensors based on conducting polymers, Sensors 15 (2015) 3801–3829.

[10] Luo, T.M.H. Lee, I.M. Hsing, Immobilization-free sequence-specific electrochemical detection of DNA using ferrocene-labeled peptide nucleic acid, Anal. Chem. 80 (2008) 7341–7346.

[11] X. Luo, I.M. Hsing, Immobilization-free electrochemical detection of DNA mutation on a microchip, Proc. μTAS'09 (2009) 33–34.

[12] T. Defever, M. Druet, D Evrard, D. Marchal, B. Limoges, Real-time electrochemical PCR with a DNA intercalating redox probe, Anal. Chem. 83 (2011) 1815–1821.

[13] (a) H.A. Wagenknecht (Ed.), Charge Transfer in DNA: From Mechanism to Applications, Wiley, New York, 2005;
(b) K.E. Augustyn, V.C. Pierre, J.K. Barton, Metallointercalators as Probes of DNA Recognition and Reactions, Wiley, New York, 2008.

[14] F. Puntoriero, S. Campagna, M.L. Di Pietro, A. Giannetto, M. Cusumano, Luminescence of a Pt(II) complex in the presence of DNA. Dependence of luminescence changes on the interaction binding mode, Photochem. Photobiol. Sci. 6 (2007) 357–360 (and Refs. therein).

[15] S. Campagna, M. Cavazzini, M. Cusumano, M.L. Di Pietro, A. Giannetto, F. Puntoriero, S. Quici, Luminescent Ir(III) complex exclusively made of polypyridine ligands capable of intercalating into calf-thymus DNA, Inorg. Chem. 50 (2011) 10667–10672 (and Refs. therein).

[16] E. Trovato, M.L. Di Pietro, F. Puntoriero, Shining a new light on an old game – an OsII-based near-IR light switch, Eur. J. Inorg. Chem. (2012) 3984–3988 (and Refs. therein).

[17] E.M. Kober, J.V. Caspar, B.P. Sullivan, T. Meyer, Synthetic routes to new polypyridyl complexes of osmium(II), Inorg. Chem. 27 (1988) 4587–4598.

[18] S. Conoci, A. Mascali, F. Pappalardo, Synthesis, DNA binding properties and electrochemistry to an electrode-bound DNA of a novel anthracene–viologen conjugate, RSC Adv. 4 (2014) 2845–2850.

[19] J.D. McGhee, P.H. von Hippel, Theoretical aspects of DNA–protein interactions: cooperative and non-co-operative binding of large ligands to a one-dimensional homogeneous lattice, J. Mol. Biol. 86 (1974) 469.