Short Communication

Biosensor for Hepatitis B Virus DNA PCR Product and Electrochemical Study of the Interaction of Di(2,2′-bipyridine) osmium(III) with DNA

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Abstract
The strategy for electrochemical detection of HBV DNA PCR product (181 bps) was designed by covalently immobilizing single-stranded HBV DNA on preoxidized glassy carbon electrode surface. The immobilization of single stranded DNA was verified by AC impedance spectra. The following hybridization reaction on surface was evidenced by electrochemical methods using $[Os(bpy)_2Cl_2]^+$ as an electroactive indicator. The interactions of $[Os(bpy)_2Cl_2]^+$ with calf thymus single and double stranded DNA immobilized on preoxidized glassy carbon electrodes were studied. $[Os(bpy)_2Cl_2]^+$ could bind preferentially to the duplex DNA by intercalating to base pairs. The intrinsic binding constant of $[Os(bpy)_2Cl_2]^+$ with calf thymus DNA was calculated to be $1.21 \times 10^4\ M^{-1}$. Using $[Os(bpy)_2Cl_2]^+$ as an electrochemical hybridization indicator, the HBV DNA sensor has been used to detect qualitatively target HBV DNA in solution with high sensitivity and selectivity.

Keywords: Biosensors, DNA, Glassy carbon electrode, Osmium complex, AC impedance, Hybridization

Electrochemical biosensors hold an important position combining the analytical power of electrochemistry with the specificity of biological recognition processes [1]. Recently, electrochemical DNA biosensors based on nucleic acid hybridization are rapidly being developed due to their increasing importance in the diagnosis of disease. DNA biosensors for recognition of DNA hybridization offer a new approach for fast, simple and inexpensive analysis of nucleic acid samples [2]. These sensors can be prepared by immobilizing single-stranded DNA (ssDNA) probes on different electrodes and using electroactive indicators to measure the hybridization events between the DNA probes and their complementary DNA (cDNA) fragments. The transition metal complexes such as $[Os(bpy)_3]^{2+}$, $[Co(phen)_3]^{3+}$ and $[Co(bpy)_3]^{3+}$) [3–5] and intercalating organic compounds (e.g., acridine orange [6]) are often used as DNA hybridization electroactive indicators. These indicators interact with ssDNA and double stranded DNA (dsDNA) by electrostatic binding to phosphate groups, hydrophobic binding to a minor groove and/or intercalation to base pairs. The difference between the binding ability of the indicator with ssDNA and dsDNA results in a difference in electrochemical responses, which produces a hybridization signal [7].

Almost 20 years ago, Palecek and his co-worker [8–10] found that osmium tetroxide complexes with pyridine (Os, py) could be covalently bound to pyrimidine bases in ssDNA, which could be detected by catalytic current on a mercury drop electrode at the potential of -1.2 V (against SCE). Os, py, thus, became one of the first chemical probes of the DNA structure [11–13]. Later, other ligands such as 2,2′-bipyridine (bpy) [12] and 1,10-phenanthroline [14] were used instead of pyridine providing better stability of the complex and extending the abilities of the probe for its application in cells [12]. Bard et al. [15] investigated the interaction of $Os(bpy)_3^{3+}$ with calf thymus DNA homogeneously by both voltammetry and ECL. Maruyama et al. [16] found that the introduction of electron-donating groups (such as $-NH_2$, $-CH_3$, and $-COOH$) could make it possible to obtain an applicable redox potential and high DNA-binding affinity for DPPZ-type osmium complex (DPPZ, dipyrido[3,2-*a*:2′,3′-*c*]phenazine), and that $[Os(DA\text{-}bpy)_2DPPZ]^{2+}$ complex (DA-bpy, 4,4′-diamino-2,2′-bipyridine) had a lower half-wave potential and higher binding affinity with DNA than those of other complexes. At present Os,L complexes have become versatile probes of the DNA structure analysis in vitro and in vivo [17].

Hepatitis B virus (HBV) is one of the causative agents of viral hepatitis and may lead to chronic hepatitis, cirrhosis, and primary liver cancer. Considerable interest has been focused on developing reliable methods for detecting and quantifying HBV DNA [18–23]. One of the most sensitive methods is to use target amplification techniques such as PCR [21–23], which has been a routine method to amplify the HBV DNA. The other method recently reported by Wang is to use the amplification feature of enzyme labels [18]. Here we describe an electrochemical biosensor using $[Os(bpy)_2Cl_2]^+$ as a hybridization indicator for the detection

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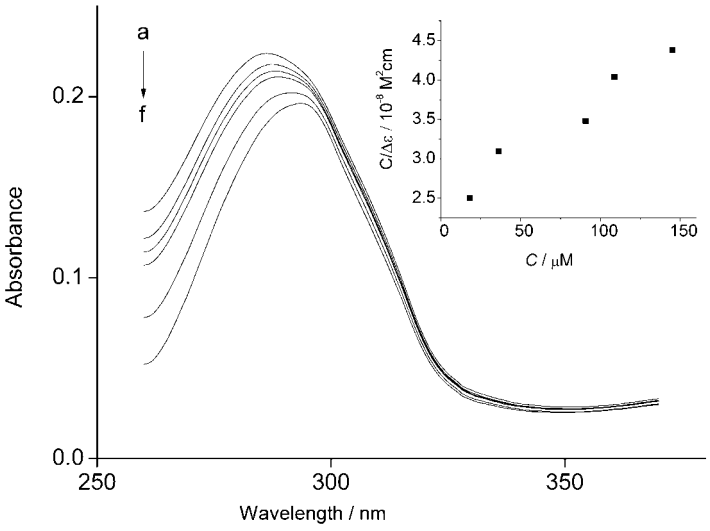

Fig. 1. Titration electronic absorption spectra of 8.33 μM $[Os(bpy)_2Cl_2]^+$ by calf thymus dsDNA: a) 0, b) 18.1, c) 36.3, d) 54.4, e) 90.7 and f) 145.1 μM. Insets: half-reciprocal plot of $[Os(bpy)_2Cl_2]^+$ with dsDNA concentration.

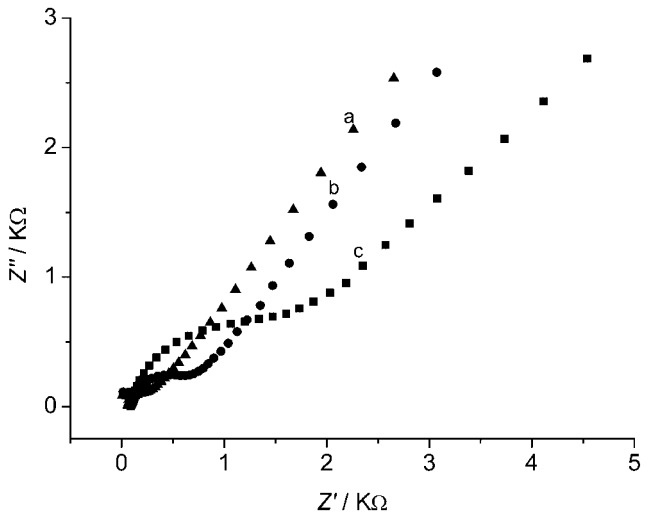

Fig. 2. AC impedance spectra of GCE(ox) (a), ssDNA/GCE(ox) (b) and dsDNA/GCE(ox) (c) in 0.5 M KNO_3 solution containing 1.0 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ (1:1) with an amplitude of 10 mV and a frequency change over the range of 10^6–0.1 Hz.

of HBV DNA fragments, which are amplified by PCR method. The proposed electrochemical method can detect about 5×10^3 copies (about 8.3×10^{-21} mol) of original genomic HBV DNA. The interaction mechanism of the osmium complexes $[Os(bpy)_2Cl_2]^+$ with calf thymus DNA is studied. The results show $[Os(bpy)_2Cl_2]^+$ can bind preferentially to the duplex DNA by intercalating to base pairs.

The electronic absorption spectra of $[Os(bpy)_2Cl_2]^+$ in the presence of increasing amounts of calf thymus dsDNA showed a decrease in the peak intensity along with a red-shift in peak position (Fig. 1). Upon addition of 145.1 μM dsDNA to 8.33 μM $[Os(bpy)_2Cl_2]^+$ solution, the absorbance of $[Os(bpy)_2Cl_2]^+$ decreased by about 12.3% and the maximum absorbance wavelength shifted to 294 nm from 286 nm. The hypochromism and red shift were the basic characteristics of intercalation on DNA structure [24, 25]. Thus, the interaction of $[Os(bpy)_2Cl_2]^+$ with calf thymus dsDNA is an intercalation.

Absorbance data of $[Os(bpy)_2Cl_2]^+$ at 286 nm with different concentrations of DNA were used to obtain the intrinsic binding constant (K_a) by Eq.(1) [26]:

$$c/\Delta\varepsilon_a = c/\Delta\varepsilon + 1/\Delta\varepsilon K_a \quad (1)$$

where c is the concentration of DNA determined spectrophotometrically assuming $\varepsilon_{260} = 6600\ M^{-1}\,cm^{-1}$ [27, 28], $\Delta\varepsilon_a = \varepsilon_a - \varepsilon_f$, and $\Delta\varepsilon = \varepsilon_b - \varepsilon_f$. ε_a, ε_b and ε correspond to the apparent extinction coefficient of $[Os(bpy)_2Cl_2]^+$, the extinction coefficient of the bound form of $[Os(bpy)_2Cl_2]^+$ by DNA and the free $[Os(bpy)_2Cl_2]^+$, respectively. The plot of $c/\Delta\varepsilon_a$ vs. c showed a linear relation with a relation coefficient of 0.9754 (inset in Fig. 1). Then the extinction coefficient of the bound form of $[Os(bpy)_2Cl_2]^+$ ($\varepsilon_b = \varepsilon_f - \Delta\varepsilon$) by calf thymus dsDNA was calculated to be $2.29 \times 10^4\ M^{-1}\,cm^{-1}$ and the intrinsic binding constant of $[Os(bpy)_2Cl_2]^+$ with calf thymus dsDNA was $1.21 \times 10^4\ M^{-1}$.

AC impedance was used to analyze the impedance change of the electrode surface during the modification process. Figure 2 shows the complex plane diagrams (Z'' vs. Z', Nyquistplot) of 1.0 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ (1:1) in 0.5 M KNO_3 solution at three electrodes. Maeda et al. [29] suggested that the calf thymus DNA immobilized on the electrode blocked the electrochemical reaction by an electrostatic repulsion between the polyanionic DNA and the anionic redox couple ions. The larger resistances at the modified electrodes are observed due to the repulsion between the negatively charged probe ions and the negatively charged phosphate groups of the DNA backbone. This result confirms the immobilization of ssDNA and hybridization of immobilized ssDNA with cDNA on the electrode. Figure 3 shows the Nyquist plots of 0.5 mM $[Os(bpy)_2Cl_2]^+$ in 10 mM Tris-HCl buffer (pH 7.0) with 20 mM NaCl at GCE(ox), calf thymus ssDNA/GCE(ox) and dsDNA/GCE(ox). The modified electrodes show lower resistances. These results are completely different from those obtained with a ferrocyanide/ferricyanide redox couple, due to their different interaction mechanisms with DNA. The osmium complex, $[Os(bpy)_2Cl_2]^+$, with positive charge can be preconcentrated at the ssDNA/GCE(ox) electrode due to the electrostatic affinity, resulting in a lower value of electron transfer resistance and a faster electron transfer rate. After hybridization of immobilized ssDNA with cDNA, the lower resistance at the dsDNA/GCE(ox) electrode is caused by the intercalation of $[Os(bpy)_2Cl_2]^+$ into the hydrophobic environment of the calf thymus dsDNA.

The electrochemical behavior of $[Os(bpy)_2Cl_2]^+$ on modified electrodes was further investigated by using differential pulse voltammetric experiments (Fig. 4). The peak current

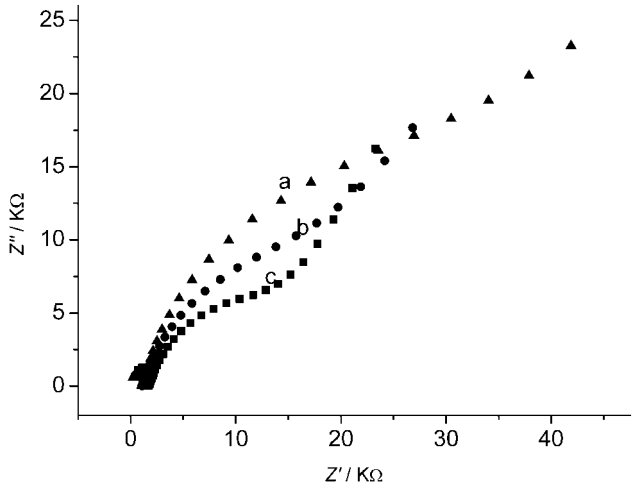

Fig. 3. AC impedance spectra of GCE(ox) (a), ssDNA/GCE(ox) (b) and dsDNA/GCE(ox) (c) in 10 mM Tris-HCl buffer (pH 7.0) with 20 mM NaCl containing 0.5 mM $[Os(bpy)_2Cl_2]^+$ with an amplitude of 10 mV and a frequency change over the range of $10^6 - 0.1$ Hz.

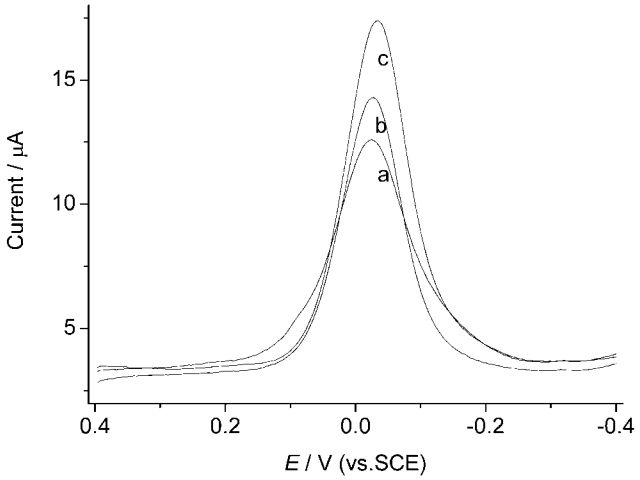

Fig. 4. Differential pulse voltammograms of GCE(ox) (a), ssDNA/GCE(ox) (b) and dsDNA/GCE(ox) (c) in 10 mM Tris-HCl buffer (pH 7.0) with 20 mM NaCl containing 0.5 mM $[Os(bpy)_2Cl_2]^+$ from -0.4 to $+0.4$ V at 20 mV s^{-1}.

of 0.5 mM $[Os(bpy)_2Cl_2]^+$ at the oxidized electrode was 8.9 μA and the peak potential was at -0.024 V. The peak currents at calf thymus ssDNA and dsDNA electrodes were 11.0 μA and 13.8 μA, respectively. The relative standard deviations of current detections for four times at the same electrodes were less than 3.0%. The increased currents by 2.1 and 4.9 μA were derived from the concentration of $[Os(bpy)_2Cl_2]^+$ at the electrode surface through association with DNA. $[Os(bpy)_2Cl_2]^+$ attached more selectively to dsDNA than ssDNA. The selectivity of $[Os(bpy)_2Cl_2]^+$ for calf thymus dsDNA was estimated as about 2.35 [30].

These results obtained on these electrode surfaces supported the conclusion of the intercalation of $[Os(bpy)_2Cl_2]^+$ into DNA, which was preciously obtained in the electronic absorption spectra. In comparison with that at the GCE(ox) electrode, the cathodic peak potentials at both ssDNA/GCE(ox) and dsDNA/GCE(ox) electrode shifted toward the negative direction (4 and 8 mV negatively shifted, respectively). Electrostatic interaction that involved an outer anionic coat along the DNA backbone can lead to negative shift in the peak potential [30]. Thus, the binding of the $[Os(bpy)_2Cl_2]^+$ to nucleic acids was related not only to the intercalation into the hydrophobic state of the dsDNA but also to the electrostatic interaction with phosphate groups of the DNA backbone. $Os(bpy)_3^{2+}$ is structurally similar to $[Os(bpy)_2Cl_2]^+$ and presumably binds to DNA mainly by electrostatic attraction, though the possibility of partial intercalation of one of the bipyridyl ligands cannot be totally dismissed [31, 33]. $[Os(bpy)_2Cl_2]^+$ is one bipyridyl ligand less than $Os(bpy)_3^{2+}$, the lower steric hindrance of $[Os(bpy)_2Cl_2]^+$ may be favorable to the intercalation into DNA. Thus one bipyridyl ligand intercalates into the hydrophobic state of the dsDNA, and the other is possibly out of the DNA backbone electrostatic interacting with the phosphate groups. However, more research still should be carried out to demonstrate this conclusion.

Because $[Os(bpy)_2Cl_2]^+$ recognized dsDNA more selectively than ssDNA, it could be applied for monitoring the hybridization event of HBV DNA fragments (Fig. 5). The peak currents of $[Os(bpy)_2Cl_2]^+$ at the HBV dsDNA/GCE(ox) electrode obtained with a hybridization process of the immobilized HBV ssDNA fragments with its cDNA obviously increased. Compared with those at the HBV ssDNA/GCE(ox) electrode, the anodic and cathodic peak currents at the HBV dsDNA/GCE(ox) electrode increased by about 22.8% and 26.9%, respectively. In contrast, the HBV DNA sensor was treated with the hybridization buffer containing 1.0 mg mL^{-1} calf thymus ssDNA for the selectivity study in the same way as in the hybridization process. No increase in peak current of the voltammogram was observable. Thus, the target sequence recognition of the sensor possesses high selectivity using $[Os(bpy)_2Cl_2]^+$ as an electroactive indicator.

PCR amplification used 10.0 μL of 1×10^7 copies of HBV DNA fragment of 181 bps as template to obtain 100 μL product solution. Then 0.1μL of the product solution was diluted to 1.0 mL with TE buffer. Half the diluted solution was used to prepare the HBV DNA biosensor, and the other was added to hybridization buffer for its target sequence recognition. Thus, the original genomic HBV DNA fragment contained in the buffer hybridization buffer was about 5×10^3 copies, which corresponded to 8.3×10^{-21} mol. Such a level of about 8.3×10^{-21} mol could be detected, indicating that the combination of electrochemical method with PCR technique possessed a high sensitivity.

In conclusion, the ssDNA was covalently immobilized via carboxylate ester linkage between 3′-hydroxy end of DNA and carboxyl group. The immobilization of ssDNA is verified by AC impedance. An electrochemical hybridization biosensor has been prepared for the sequence-selective detection of short stranded HBV DNA using $[Os(bpy)_2Cl_2]^+$ as an electroactive indicator. The binding of $[Os(bpy)_2Cl_2]^+$

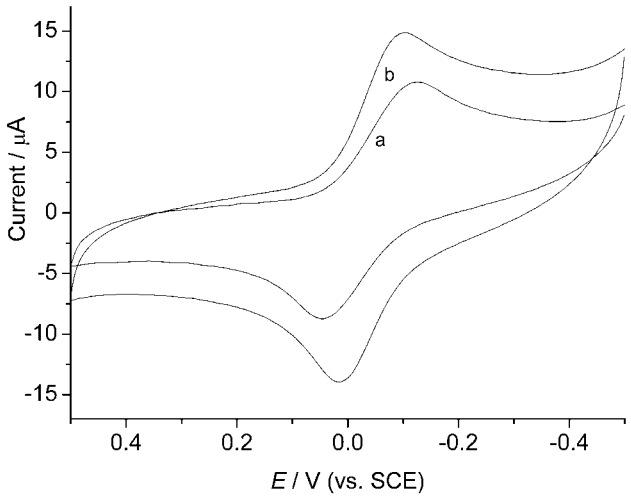

Fig. 5. Cyclic voltammograms of HBV ssDNA/GCE(ox) (a) and HBV dsDNA/GCE(ox) (b) in 10 mM Tris-HCl buffer (pH 7.0) with 20 mM NaCl containing 0.5 mM $[Os(bpy)_2Cl_2]^+$ at 100 mV s^{-1}.

to DNA is related not only to the intercalation into the hydrophobic state of the dsDNA but also to the electrostatic interaction with phosphate groups of the DNA backbone. The electrochemical biosensor combining with PCR method can provide a highly sensitivity detection of about 8.3×10^{-21} mol of original genomic HBV DNA.

Experimental

HBV DNA fragments (base location: 486 – 666) were amplified by PCR method using oligonucleotide primers 5′-CAT CAT CCT GGG CTT TC-3′ and 5′-AAA AAG GGA CTC AAG ATG TTG TA-3′ according to the procedure reported preciously [34]. Denatured ssDNA was produced by heating native dsDNA in a water bath at 100 °C for about 5 min followed by rapid cooling in an ice bath.

The absorption titrations with DNA were conducted by keeping the concentration of $[Os(bpy)_2Cl_2]^+$ (Aldrich) at a constant value and varying the nucleic acid concentration. This was carried out by adding 10 μL DNA stock solution to samples each time and the volume effect could be ignored. The corresponding concentrations of DNA were used as contrast solutions.

Glassy carbon electrodes (3 mm diameter) were then oxidized at + 1.5 V for 10 s in an aqueous solution containing 2.5% $K_2Cr_2O_7$ and 10% HNO_3. After being rinsed the electrode, named as GCE(ox), was dipped in 0.1 M phosphate buffer (pH 7.0) containing 1 mg/mL calf thymus ssDNA or in 0.5 mL TE solution containing 0.1 μL PCR product of HBV ssDNA and 0.04 mg EDAC (Sigma) for 1 h. Thus the ssDNA was immobilized on the oxidized glassy carbon electrode. Afterward the electrode was washed with 0.1% (m/m) SDS phosphate buffer (pH 7.0) and then water. It was stored in 10 mM Tris-HCl buffer (pH 7.0) with 20 mM NaCl at 4 °C.

An aliquot of 0.5 mL of 0.3 M NaCl + 0.03 M sodium citrate buffer containing 0.05 μL DNA PCR product or 1 mg/mL calf thymus DNA as control was reheated in a 100 °C water bath for about 5 min, then cooled slowly to about 55 °C. The ssDNA electrode was immersed into the solution, and incubated in a water bath at 42 °C for 1 h to form dsDNA at the electrode surface. After hybridization, the dsDNA electrode was washed with 0.1% (m/m) SDS phosphate buffer (pH 7.0) and then water.

The absorption spectra were recorded on a Lambda 35 UV/vis spectrometer (Perkin Elmer Co, USA). The AC impedance experiments were performed on an AUTOLAB analyzer (EcoChemie, Netherlands). The cyclic voltammetric experiments (CV) and differential pulse voltammetric (DPV) experiments were carried out with CHI 660 Electrochemical Workstation (CH Instruments Inc., USA). Electrochemical experiments were carried out at room temperature with a three-electrode system consisting of a platinum wire as counter electrode, a saturated calomel electrode (SCE) as reference and an oxidized glass carbon or DNA modified electrode as the working electrode.

Acknowledgements

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