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Hybridization biosensor using di(2,2′-bipyridine)osmium (III) as electrochemical indicator for detection of polymerase chain reaction product of hepatitis B virus DNA

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Abstract

A novel hepatitis B virus (HBV) DNA biosensor was developed by immobilizing covalently single-stranded HBV DNA fragments to a gold electrode surface via carboxylate ester to link the 3′-hydroxy end of the DNA with the carboxyl of the thioglycolic acid (TGA) monolayer. A short-stranded HBV DNA fragment (181 bp) of known sequence was obtained and amplified by PCR. The surface hybridization of the immobilized single-stranded HBV DNA fragment with its complementary DNA fragment was evidenced by electrochemical methods using $[Os(bpy)_2Cl_2]^+$ as a novel electroactive indicator. The formation of double-stranded HBV DNA on the gold electrode resulted in a great increase in the peak currents of $[Os(bpy)_2Cl_2]^+$ in comparison with those obtained at a bare or single-stranded HBV DNA-modified electrode. The mismatching experiment indicated that the surface hybridization was specific. The difference between the responses of $[Os(bpy)_2Cl_2]^+$ at single-stranded and double-stranded DNA/TGA gold electrodes suggested that the label-free hybridization biosensor could be conveniently used to monitor DNA hybridization with a high sensitivity. X-ray photoelectron spectrometry technique has been employed to characterize the immobilization of single-stranded HBV DNA on a gold surface.

Keywords: Electrochemical DNA biosensor; Polymerase chain reaction; Hepatitis B virus; Hybridization; $[Os(bpy)_2Cl_2]^-$

Biomolecular recognition has been used extensively for analytical purposes. Recently, DNA biosensors based on nucleic acid hybridization, one of the most important biomolecular recognition processes, are rapidly being developed due to their increasing importance in the diagnosis of disease. Electrochemical DNA biosensors for recognition of DNA hybridization have been attracting increased attention [1] because of their advantages, such as low cost, simple design, small dimensions, and low power requirements [2]. These sensors can be prepared by immobilizing single-stranded DNA (ss-DNA)[1] probes on different electrodes and using electroactive indicators to measure the hybridization events between the DNA probes and their complementary DNA fragments. In general, there are two kinds of DNA hybridization electroactive indicators: metal coordination complexes and intercalating organic compounds. Transition metal complexes are often used as the electroactive indicators of DNA sensors, include Ru, Co, Fe, and Os complexes [3–7]. These complexes consist of one or several transition metal ion(s) as center ion(s) and several organic heterocycles as ligands. The good electrochemical properties of the center ion(s) and

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[1] *Abbreviations used:* CT, calf thymus; ds-DNA, double-stranded DNA; HBV, hepatitis B virus; ss-DNA, single-stranded DNA; TE buffer, Tris–ethylenediamine tetraacetic buffer; TGA, thioglycolic acid; XPS, X-ray photoelectron spectrometry; Os,Py, osmium tetroxide complex with pyridine.

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the intercalative or groove interaction between heterocycles and DNA helix make the hybridization events easily transformable into electrochemical signals. The study on the interaction between metal complexes and DNA is of great importance for DNA recognition and hybridization monitoring [8,9]. Several methods, such as luminescence analysis [10–13], electrophoresis [14], nuclear magnetic resonance [15,16], quartz-crystal microbalance [17,18] and electrochemical analysis [6,19–21], have been used to examine the DNA-binding properties with the metal complex indicators.

In the early 1980s, Paleček and his co-workers found that osmium tetroxide complexes with pyridine (Os,py) could react with pyrimidine moieties in ss-DNA, yielding stable adducts at the 5,6 double bond in the pyrimidine ring, which could be detected by catalytic current on a mercury drop electrode at the potential of −1.2 V (vs saturated calomel electrode). In B-form ds-DNA the reaction with Os,py or Os,bpy (bpy, bipyridine) was sterically blocked, which made these species suitable for probing of open DNA structures involving unpaired bases reactive toward the osmium tetroxide complexes [20,21]. Studies of Ropp and Thorp showed that the nucleobase 8-oxoguanine was selectively oxidized by the redox catalyst $Os(bpy)_3^{3+/2+}$ in the presence of native guanine; this site-selective mismatch sensitivity could be applied to the detection of mononucleobase difference [22]. Maruyama et al. found that the introduction of electron-donating groups (such as $-NH_2$, $-CH_3$, and –COOH) could effectively control the redox potential of DPPZ-type osmium complex (DPPZ, dipyrido[3,2-*a*:2′,3′-*c*]phenazine), and $[Os(DA\text{-}bpy)_2DPPZ]^{2+}$ 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 [23]. Thus, osmium complexes are always used to modify DNA to form DNA–Os,L complexes that can be quantitatively detected by electrochemical methods [24–26].

In this work, $[Os(bpy)_2Cl_2]^+$ is used as a novel electrochemical hybridization indicator for monitoring the hybridization event of HBV DNA fragments which are amplified with a PCR method. The amplified HBV ss-DNA fragments are first bound to a TGA self-assembled monolayer on a gold electrode surface and then hybridized with their target sequence, the HBV cDNA, to form a ds-DNA-modified surface. As control, a CT ss-DNA-modified electrode is prepared for the selectivity study of the developed HBV DNA biosensor.

Persistent infection with HBV is a major health problem worldwide and may lead to chronic hepatitis, cirrhosis, and primary liver cancer [27]. The detection of HBV DNA in the serum of patient is an important tool in the diagnosis of HBV infection. Of the different methods available to detect serum HBV DNA, the most sensitive is to use target amplification techniques, such as PCR [28–31], which has been used as a routine method to amplify the HBV DNA target. The proposed electrochemical method can detect about 5×10^3 copies (about 8.3×10^{-21} mol) of original genomic HBV DNA.

Materials and methods

Reagents

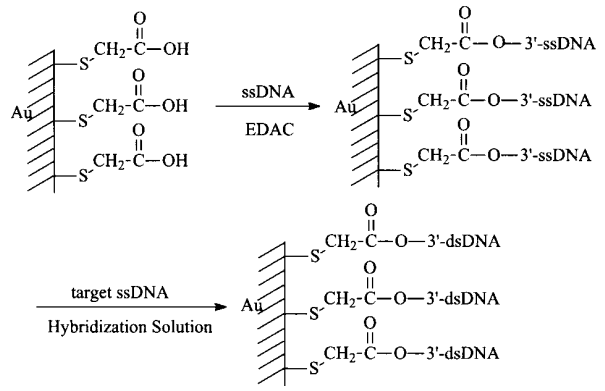
HBV genetic assay kit and marker reagents were purchased from Kehua Co. (Shanghai, China). $[Os(bpy)_2Cl_2]Cl$, thiohydracrylic acid, and gelose were obtained from Aldrich (USA). Calf thymus DNA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and tris-(hydroxymethyl)aminomethane (Tris) were purchased from Sigma (USA) and used as received. Sodium dodecyl sulfate (SDS) was from Guangyao Chemical Reagent Co. (Jiangsu, China). All other reagents were of analytical reagent grade. All solutions were made up with twice-quartz-distilled water. Native ds-DNA was dissolved in 10 mM Tris–EDTA (TE) buffer (pH 8.0) prior to use. ss-DNA was produced by heating a native ds-DNA solution in a 100 °C water bath for about 5 min and then rapidly cooling in an ice bath.

Polymerase chain reaction

PCR amplification was performed on a standard Perkin–Elmer 9600 cycles (Perkin–Elmer, Warrington, UK) using oligonucleotide primers 5′-CAT CAT CCT GGG CTT TC-3′ and 5′-AAA AAG GGA CTC AAG ATG TTG TA-3′ in 0.2-ml thin-walled polypropylene tubes. The amplification mixture, in a final volume of 100 μl, containing 10.0 μl 10× PCR buffer, 6.0 μl 25 mM $MgCl_2$, 6.0 μl 4 × 5 mM dNTPs, 3.0 μl of each primer, and 10.0 μl of 1×10^7 copies of HBV DNA as template. Before the amplification cycles, the amplification mixture was predenatured at 100 °C for 5 min and then was supplemented with 25 units *Taq* polymerase. The thermal program comprised an initial denaturation at 94 °C for 90 s followed by 40 amplification cycles. Each cycle included two steps: hold at 94 °C for 25 s and at 60 °C for 60 s. After the cycle step, an extension step was performed at 70 °C for 10 min. Finally the mixture was kept at 4 °C before use. Maximum ramp rates were used throughout. The fragments were separated by agarose gel electrophoresis to obtain 100 μl PCR product. The amplification product without further purification gave an A_{260}/A_{280} ratio of 1.82, thus the DNA was pure enough.

Immobilization of ss-DNA on the TGA self-assembled monolayer gold electrode

Gold electrodes (0.5 mm diameter) were first abraded successively with finer grades of SiC paper; then



Scheme 1. Immobilization of ss-DNA on TGA monolayer-modified Au electrode and hybridization with complementary target ss-DNA.

polished to a mirror-like surface with 0.05-μm alumina slurry on microcloth pads (Buehler), and ultrasonicated in turn with acetone and water for 3 min, respectively. The electrode was rinsed with water and modified immediately by immersing it in 0.1 mM TGA solution for 6 h to form a TGA self-assembled monolayer on the gold surface. Then the modified electrode was dipped in 200 mg/ml CT ss-DNA solution or in 0.5 ml HBV ss-DNA TE solution containing 0.05 μl PCR product and 0.04 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide for 1 h. Thus the ss-DNA was immobilized on the modified gold electrode. The covalent immobilization of ss-DNA on the electrode is illustrated in Scheme 1. The ss-DNA was covalently immobilized on the gold electrode via carboxylate ester linkages between the 3′-hydroxy end of the DNA and the carboxyl of the TGA. 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 TE buffer (pH 8.0) prior to use.

Hybridization

An aliquot of 0.5 ml of 0.3 M NaCl + 0.03 M sodium citrate buffer containing 0.05 μl DNA PCR product was reheated in a 100 °C water bath for about 5 min and then cooled slowly to about 55 °C, which took about 25 min. The HBV ss-DNA/Au electrode was immersed into the hybridization solution and incubated in a water bath at 42 °C for 1 h to form ds-DNA at the electrode surface (Scheme 1). Obviously, the original genomic HBV DNA fragment in the hybridization buffer was about 8.3×10^{-21} mol. In fact the amount of ds-DNA immobilized on the Au electrode surface was even less. As a control, the denatured CT DNA with a concentration of 0.1 mM in the hybridization buffer was treated in the same way. After hybridization, the ds-DNA electrodes were washed with 0.1% (m/m) SDS phosphate buffer (pH 7.0) and then water.

Apparatus and measurements

Electrochemical experiments were carried out at room temperature with a three-electrode system comprising a platinum wire as counter, a saturated calomel electrode as reference, and a bare gold or DNA-modified gold electrode (DNA/Au) as the working electrode. The electrodes were connected to a BAS-100B electrochemical analyzer with a PA-1 preamplifier (Bioanalytical System, Inc., USA). XPS spectra were performed on a VG ESCA-LAB MKII spectrometer (VG Co., UK) using Mg radiation.

Results and discussion

Interaction between $[Os(bpy)_2Cl_2]^+$ and immobilized CT ss-DNA or CT ds-DNA

Fig. 1 shows the cyclic voltammograms of 0.50 mM $[Os(bpy)_2Cl_2]^+$ at bare gold, CT ss-DNA/Au, and CT ds-DNA/Au electrodes at 400 mV/s. The data of peak currents and peak potentials determined from these curves are listed in Table 1. Obviously, the redox peak currents of $[Os(bpy)_2Cl_2]^+$ at the CT ss-DNA/Au electrode are slightly larger than those at the bare electrode. After the hybridization process, the peak currents at the CT ds-DNA/Au electrode increase by 31% for the

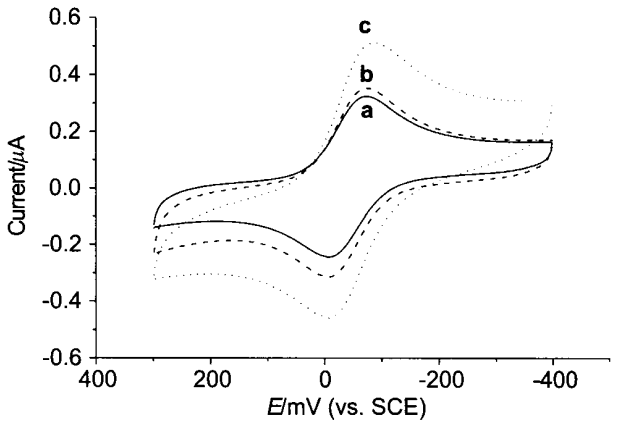


Fig. 1. Cyclic voltammograms of bare gold (a), CT ss-DNA/Au (b), and CT ds-DNA/Au (c) electrodes in 10 mM, pH 7.4, Tris–HCl buffer containing 0.50 mM $[Os(bpy)_2Cl_2]^+$ at 400 mV/s.

Table 1
Results from the cyclic voltammograms of 0.50 mM $[Os(bpy)_2Cl_2]^+$ in 10 mM, pH 7.4, Tris–HCl buffer at 400 mV/s

Electrode	$E^{0'}$/mV	ΔE_p/mV	i_{pa}/μA	i_{pc}/μA
Bare gold	−41	65	0.278	0.286
CT ss-DNA/Au	−39	67	0.294	0.291
CT ds-DNA/Au	−46	65	0.364	0.422

anodic and 48% for the cathodic currents. The greater peak currents at the ds-DNA/Au electrode than at the ss-DNA/Au electrode indicate that more $[Os(bpy)_2Cl_2]^+$ molecules are concentrated or bound to ds-DNA helix than to ss-DNA helix. Thus $[Os(bpy)_2Cl_2]^+$ can be used as an electroactive indicator for recognition of the surface hybridization process.

The formal potential $E^{0'}$ of $[Os(bpy)_2Cl_2]^+$ at three electrodes, which is obtained from the average of the reduction and oxidation peak potentials, almost stays at a constant value. At the same time, the change in the peak-to-peak separation is also very low. As is well known, the interaction mechanism between electroactive indicator and DNA helix depends on their kinds and structures. An electrostatic interaction of an electroactive indicator with ss-DNA or ds-DNA will make its reduction at the surface with negative charge more difficult and thus result in a shift of formal potential in the negative direction [32,33]. In contrast, the intercalative interaction produces a positive shift of formal potential of the intercalator [32,33]. Thus, the binding properties of $[Os(bpy)_2Cl_2]^+$ to immobilized ss-DNA or ds-DNA are not a pure electrostatic or intercalative interaction. The two contrary factors may lead to a constant $E^{0'}$ value of $[Os(bpy)_2Cl_2]^+$ at the ss-DNA/Au and ds-DNA/Au electrodes. In fact, the interaction between Os,py and DNA is unusual compared with other electroactive indicators [7,20,21,24]. Paleček and co-workers thought that Os,py could form covalent adducts with pyrimidine moieties (preferentially with thymine, displaying about 10-fold higher reactivity, compared to cytosine) in ss-DNA [20,21]. Motonaka and co-workers immobilized DNA probes with different lengths to hybridize with their targets and studied the recognition of the osmium complex, tris(bipyridyl)osmium (II/III) ion, in the hybridization process [7]. The results showed that the osmium complex interacted with major or minor grooves of the ds-DNA helix. However, the change in peak potential upon the major or minor groove interaction has not been reported. Thus it is difficult to describe exactly the interaction mechanism between $[Os(bpy)_2Cl_2]^+$ and the CT DNA helix due to the particularity of Os,py complexes. From the value of peak-to-peak separation at 400 mV/s the rate constant for the electron transfer is estimated to be $1.12\,s^{-1}$ [34]. Therefore, the electron transfer of $[Os(bpy)_2Cl_2]^+$ in both its free and its bound states at the electrode surface is fast.

Recognition by HBV DNA sensor of its target sequence using $[Os(bpy)_2Cl_2]^+$ as an electrochemical indictor

Fig. 2 indicates that the redox peak currents of $[Os(bpy)_2Cl_2]^+$ continuously increase and the peak potentials remain at constant values following the further treatment of the electrode with HBV ss-DNA fragments, showing an electrochemical behavior similar to that at

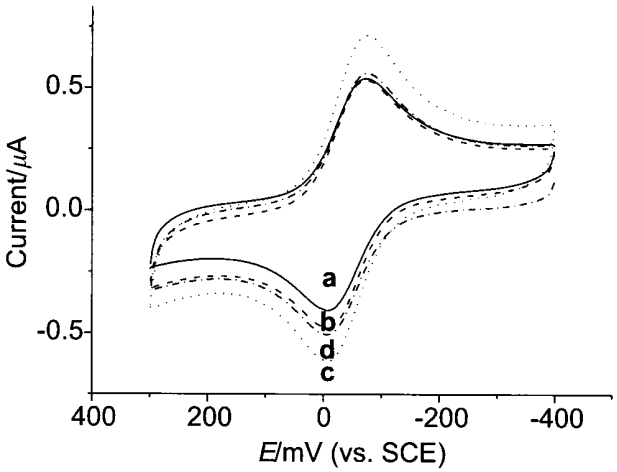


Fig. 2. Cyclic voltammograms of 0.50 mM $[Os(bpy)_2Cl_2]^+$ at bare gold (a), HBV ss-DNA/Au (b), and HBV ds-DNA/Au (c) electrodes and at HBV ss-DNA/Au electrode treated with calf-thymus ss-DNA (d) at 400 mV/s.

the CT ss-DNA and ds-DNA/Au electrodes. Thus the interactions between $[Os(bpy)_2Cl_2]^+$ and the covalently immobilized HBV ss-DNA fragment and its hybridization product show the same mechanisms as those of CT DNA.

The peak currents of $[Os(bpy)_2Cl_2]^+$ at HBV ss-DNA/Au electrode increase slightly in comparison with those at the bare gold electrode, indicating a very weak binding of the complex to the ss-DNA fragment. However, the peak currents of $[Os(bpy)_2Cl_2]^+$ at the HBV ds-DNA/Au electrode obtained with a hybridization process between the immobilized HBV ss-DNA fragment and its cDNA obviously increase. Compared with those at the bare gold electrode, the anodic and cathodic peak currents at the HBV ds-DNA/Au electrode increase by about 23 and 25%, respectively. The smaller increase in peak current at the HBV ds-DNA/Au electrode than at the CT ds-DNA/Au electrode is due to its shorter chain length or fewer binding sites, which leads to fewer $[Os(bpy)_2Cl_2]^+$ molecules bound to HBV ds-DNA/Au than to CT ds-DNA/Au. The enhancement of peak currents indicates that more $[Os(bpy)_2Cl_2]^+$ molecules are preconcentrated on the ds-DNA/Au electrode surface due to the binding interaction between $[Os(bpy)_2Cl_2]^+$ and immobilized HBV ds-DNA. Thus both the hybridization process and the target sequence of the HBV DNA fragment can be recognized using $[Os(bpy)_2Cl_2]^+$ as an electrochemical indictor.

Selectivity of HBV DNA sensor for target sequence recognition

As control, the HBV DNA sensor was treated with the hybridization buffer containing 0.1 mM CT ss-DNA for the selectivity study in the same way as in the

Table 2
Results for detection of HBV DNA PCR product with cyclic voltammetry in 10 mM, pH 7.4, Tris–HCl buffer containing 0.50 mM $[Os(bpy)_2Cl_2]^+$ at 400 mV/s

Sensor	$E^{0'}$/mV	ΔE_p/mV	i_{pa}/μA	i_{pc}/μA
HBV DNA sensor	−40	61	0.285	0.284
Hybridization with HBV cDNA	−42	63	0.343	0.356
Mismatch with CT ss-DNA	−39	63	0.275	0.285

hybridization process (curve d in Fig. 2). No increase in peak current of the voltammogram of $[Os(bpy)_2Cl_2]^+$ was observable except a slight change of background current (see the data shown in Table 2), indicating that CT ss-DNA did not bind to the immobilized HBV ss-DNA; in other words, the HBV DNA sensor could not hybridize with CT ss-DNA. Thus, the target sequence recognition of the sensor possesses high selectivity by using $[Os(bpy)_2Cl_2]^+$ as an electroactive indicator.

Sensitivity of target sequence recognition

This work uses 10.0 μl of 1×10^7 copies of HBV DNA fragment of 181 bp as template for PCR amplification to obtain 100 μl product solution. Product solution (0.1 μl) was diluted to 1.0 ml with TE buffer, and 0.5 ml diluted solution was then used to prepare the HBV DNA biosensor and 0.5 ml diluted solution was added to hybridization buffer for its target sequence recognition. Thus the original genomic HBV DNA fragment contained in the hybridization buffer was about 5×10^3 copies, which corresponds to 8.3×10^{-21} mol. Obviously, the amount of cDNA hybridized to HBV ss-DNA/Au was less than 8.3×10^{-21} mol. Such a level could result in an obvious increase in peak currents of $[Os(bpy)_2Cl_2]^+$ (curves a and c in Fig. 2), indicating that the combination of electrochemical method with PCR technique could determine the target sequence with a high sensitivity, down to 10^3 copies, or 10^{-21} mol of the original genomic fragment.

XPS spectra

XPS is a powerful tool for the identification of all the elements in the periodic table with the exception of hydrogen and helium and has been widely used for determination of the chemical state of an element and the type of species to which it is bonded. The peak position of XPS is related to the specific element [35]. The XPS spectra of both TGA/Au and HBV ss-DNA/Au electrodes (given as curves a and b, respectively, in Fig. 3) exhibited the peaks of S, C, and O elements [35] (Figs. 3-3, 3-4, and 3-6), which came from the self-assembled monolayer of TGA for the TGA/Au electrode and from both the monolayer and the immobilized ss-DNA fragment for the HBV ss-DNA/Au electrode. Curve b shows also the peaks of P

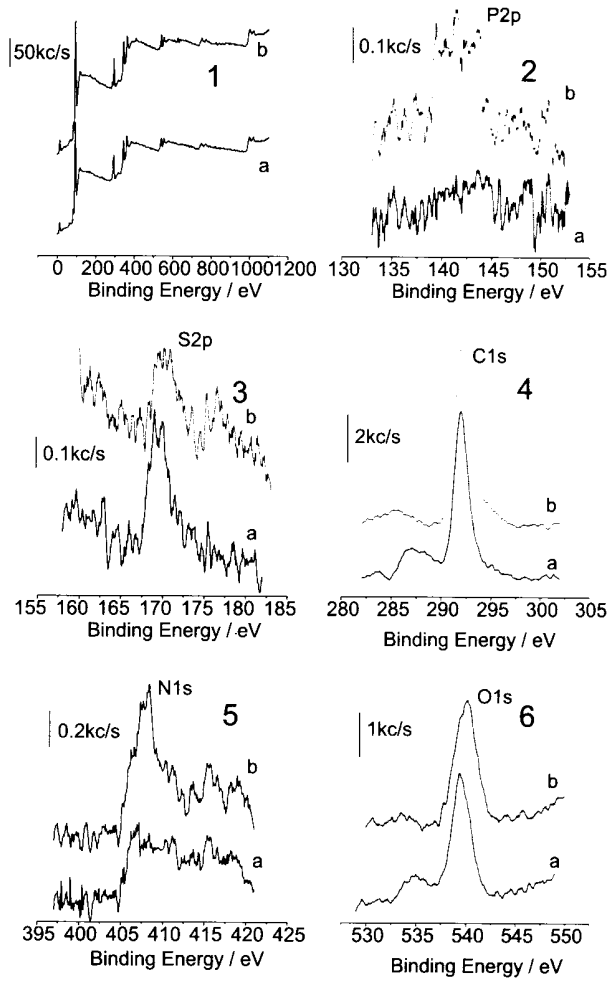


Fig. 3. XPS spectra of TGA/Au (a) and ss-DNA/Au (b) from 0 to 1200 eV (1) and individual expanded spectra from 130 to 150 eV for P2p (2), 155 to 185 eV for S2p (3), 280 to 305 eV for C1s (4), 395 to 415 eV for N1s (5), and 520 to 550 eV for O1s (6).

(Fig. 3-2) and N (Fig. 3-5) elements attributed to the immobilized HBV ss-DNA fragment. The different peak intensities of S2p at the TGA/Au and the ss-DNA/Au electrodes (Fig. 3-3) resulted from their different structures. At the ss-DNA/Au electrode the peak intensity of S2p was lower, which was attributed to the burying of the S atom in the DNA fragment.

The occurrences of P2p and N1s peaks are the best evidence that ss-DNA had been covalently immobilized on the TGA self-assembled monolayer-modified gold surface. From the peak areas of curve b in Figs. 3-5 and 3-6, the molar ratio of N–O is calculated to be 1:1.82. In the sequence-known HBV DNA fragment the ratio of (G+C):(A+T) is 49:51. Thus, the ratio value of N:O is calculated theoretically to be 1:1.87 in the HBV ss-DNA fragment. The experimental value of N:O obtained from XPS is near the theoretical value, verifying that the material immobilized on the gold electrode surface is HBV ss-DNA.

Conclusions

An electrochemical hybridization biosensor has been developed for sequence detection of PCR product of short-stranded HBV DNA fragment by using $[Os(bpy)_2Cl_2]^+$ as an electroactive indicator. The XPS spectra of TGA self-assembled monolayer and the sensor demonstrate that the immobilization of HBV ssDNA on a gold surface is successful. The label-free hybridization biosensor can be used for the detection of sequence-specific DNA with good selectivity. The combination of electrochemical method with PCR technique results in highly sensitive detection of DNA fragments.

Acknowledgments

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