BIOCHIP JOURNAL, Vol. 2, No. 1, 66-72, March 2008

Microelectrode Array for Electrochemiluminescence-based DNA Detection

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Accepted 17 February 2008

Abstract

This paper reports on the design, fabrication and testing of microelectrode arrays for electrochemiluminescence-based detection and quantitative analysis of DNA. To maximize the anode surface area in a small real estate different geometrical configurations of an anode such as wave-shaped, comb-shaped, and straight parallel coplanar arrangements with suitably designed cathode were designed. An asymmetric Ru-ligand complexe $Ru(bpy)_2(dcbpy)(PF6)_2$[bpy, 2,2′-bipyridine; dcbpy, 4,4′-dicarboxyl-2,2′-bipyridine] was used as an electro-chemiluminescing DNA label. The detection and quantification of single-stranded DNA using the developed microelectrode arrays are presented. Preliminary tests yielded a detection limit of 10 nmol of Ru (II) chelate labeled DNA with a sample volume as low as 1 μL. The microelectrode array system can be integrated with a bipolar semiconductor photodiode array chip to fabricate a highly compact embodiment for on-chip handling of solutions and electrochemiluminescence detection.

Keywords: Electrochemiluminescence, Microelectrode, DNA

Introduction

Recently miniaturized point-of-care-testing (POCT) devices have drawn much interest in bio-analytical research due to their unique characteristics such as near-patient and decentralized testing[1]. POCT is potentially unrestricted to location, personnel, or test menu. The POCT idea can be efficiently utilized for the qualitative and quantitative detection of biomolecules, including proteins, nucleic acids etc. Thus, an A POCT system, able to perform from detection to analysis, should preferably be a portable, miniaturized, integrated device.

Advances in electronic signal detection technologies have contributed greatly to the miniaturization of POCT devices, making them more convenient for high-throughput screening with less sample consumption. Recently, biosensors based on integrated circuit (IC) microchips with complementary metal oxide semiconductor (CMOS) photocell arrays for optical detection have been developed[2-4]. The miniaturized CMOS microchip system integrates photosensors, amplifiers, and logic circuits in a single IC package. Detection of a single bacterium using a sensitive laser-induced fluorescence method has been demonstrated in a CMOS-based biochip system[5]. Alternatively, a two-dimensional photodiode array (PDA) microchip-based on-chip detection technology can be another miniaturized platform for bioanalytical measurements. Our group has developed an IC PDA microchip using a conventional bipolar semiconductor technology[6-9]. The chip can act as a sample platform as well as a two-dimensional photodetector. Unlike CMOS devices-, which are driven by voltage the bipolar PDA chip device is current driven. This property of the bipolar microchip offers greater advantage for linear amplification of current produced by a photodiode because the output signal of the photodiode is also current. Successful applications of the developed PDA chip for high-throughput drug screening based on cell viability measurements, and on-chip detection of DNA and protein, have been reported[6-9]. The unique design empowered our chip system with excellent properties, such as high-performance analog signal processing at a low noise level. Since a PDA microchip can be used as a sample platform wherein it serves as a highly sensitive detector itself[9], the need for additional bulky detectors such as a microscope or charge coupled device (CCD) as used in other miniaturized detection systems can easily be eliminated.

The PDA system contained a light emitting diode (LED) as a light source. The principle of detection methodology was based on an enzymatic on-chip reaction and light absorption property of enzymatic reaction products, or dye stained cells. For further miniaturization of the detection device it is desirable to remove the external light source, such as LED or laser. Detection of electrochemiluminescence (ECL)

from an assay using a bipolar microchip will undoubtedly eliminate the need of an external light source, thus making it another attractive choice as a POCT detection technology. Likewise, if the sensitivity of the photodiodes is properly tuned a more miniature as well as more sensitive detection system can be constructed.

The ECL process, in which highly reactive species are generated from stable precursors at the surface of an electrode, occurs with numerous molecules including compounds of ruthenium, e.g. Chemiluminescent reactions between highly reactive species that lead to the emission of light are initiated electrically, rather than chemically. ECL-based DNA probe assays necessarily start with labeling the oligonucleotides with an electrochemiluminescent label. An efficient metal-based label should be resistant to heat and chemical challenges, and offer ease of labeling and lack of interference from endogenous metal ions.

In this paper we report on the design and testing of a microelectrode array that in an eventual embodiment can be integrated with a bipolar PDA microchip, allowing on-chip electrochemiluminescence detection. An asymmetric Ru-ligand complexe $Ru(bpy)_2(dcbpy)(PF6)_2$[bpy, 2,2′-bipyridine; dcbpy, 4,4′-dicarboxyl-2,2′-bipyridine] was used as a luminescent probe to label the oligonucleotides. To illustrate the usefulness of the microelectrodearrays Ru (II)-labeled oligonucleotides were quantitatively detected.

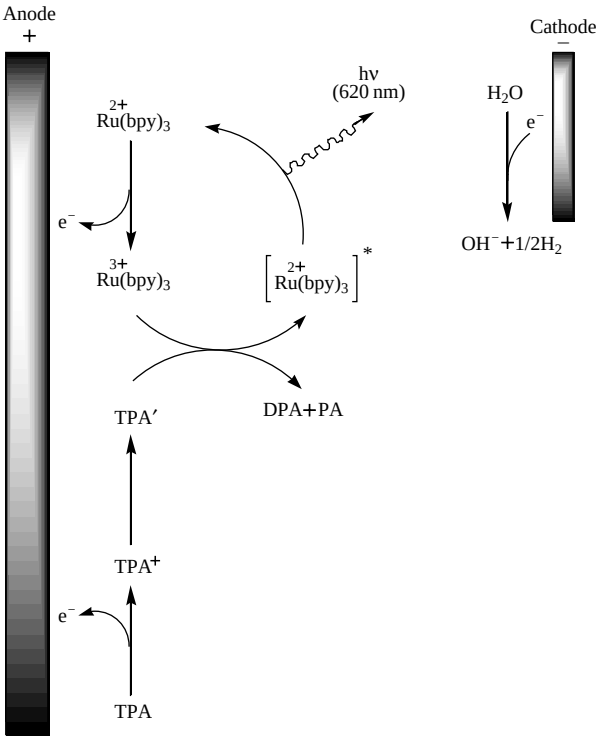

Figure 1. Diagram of the electrochemical oxidation and chemical reduction reactions for $[Ru(bpy)_3]^{2+}$)+TPA (*Ref.* 10).

Results and Discussion

Principle of Electrochemiluminescence

The development of ECL bioassays is based on the use of a ruthenium complex and tripropylamine (TPA). The potentials required for electrochemical reaction are relatively low (1-2 *V*), making ECL generation very compatible with microelectronic circuits. The electrochemistry of the reaction between TPA and tris (2,2′-bipyridyl) ruthenium (II) (TBR)[10] is shown in Figure 1. Initially, $Ru(bpy)_3^{2+}$ and TPA are oxidized at approximately the same voltage at the anode surface. TPA^+ immediately undergoes deprotonation to form a powerful reducer, TPA′, that chemically reacts with $Ru(bpy)_3^{3+}$, and $Ru(bpy)_3^{3+}$ enters an excited state, $[Ru(bpy)_3^{2+*}]$, by a high-energy electron transfer from the electron carrier. Relaxation of the resulting TBR molecule to its ground state by emitting a photon results in a light emission at 620 nm.

It is noteworthy that while TPA decomposes to dipropyl amine and is therefore consumed in this reaction, $Ru(bpy)_3^{2+}$ is not consumed during the reaction and may be oxidized and excited repeatedly, if there is excessive TPA in the buffer[11-13]. Luminescence occurs at the surface of the anode. Assuming that the component of the anode current due to oxidation of TBR is diffusion limited[14], it can be proportionally correlated to the bulk Ru (II) complex concentration. Since the luminescence is directly proportional to the concentration of the oxidized Ru (II) probe, the luminescence intensity should be proportional to its bulk concentration. The high ECL efficiency of $Ru(bpy)_3^{2+}$ and its low oxidation potential make it very attractive for microsensor applications.

Synthesis of ECL Probes and Labeling of Oligonucleotides

In order to use ECL in the quantification of DNA, the DNA molecule of interest must be selectively labeled with an electroluminescent label. Various approaches have been developed to label DNA with ruthenium chelate. IGEN, Inc. (Rockville, MD), developed a phosphoramidite of tris(2,2′-bipyridine) ruthenium (II) complex, bis(2,2-bipyridine){4-[4-(2-cyanoethoxy-N,N-diisopropylamino)phosphinoxybutyl]4′-methyl}2,2-bipyridine ruthenium (II) dihexafluorophosphate, which is available commercially under the brand name of Origen Phosphoramidite[15].

These complexes were initially developed as N-hydroxysuccinamide ester (NHS) by the same research group[11,16]. Other Ru (II) complexes such as those with bathophenanthroline ligands were also synthesized[17,18] as time-resolved fluorescent labels. Origen Phosphoramidite products are patent-protected. In 1992, Igen licensed to Boehringer Mannheim (BMG; Mannheim, Germany) its ECL-based technology (Origen)[19]. At present BioVeris (Gaithersburg, MD) and its licensees provide products that are based on its unique patent-protected ECL technology to the global diagnostics markets.

In the present paper, we utilized metal-ligand complexes to evaluate the efficacy of microelctrode arrays to quantify DNA based on ECL. Metal-ligand complexes (MLC) such as tris(2,2′-bipyridine)ruthe-

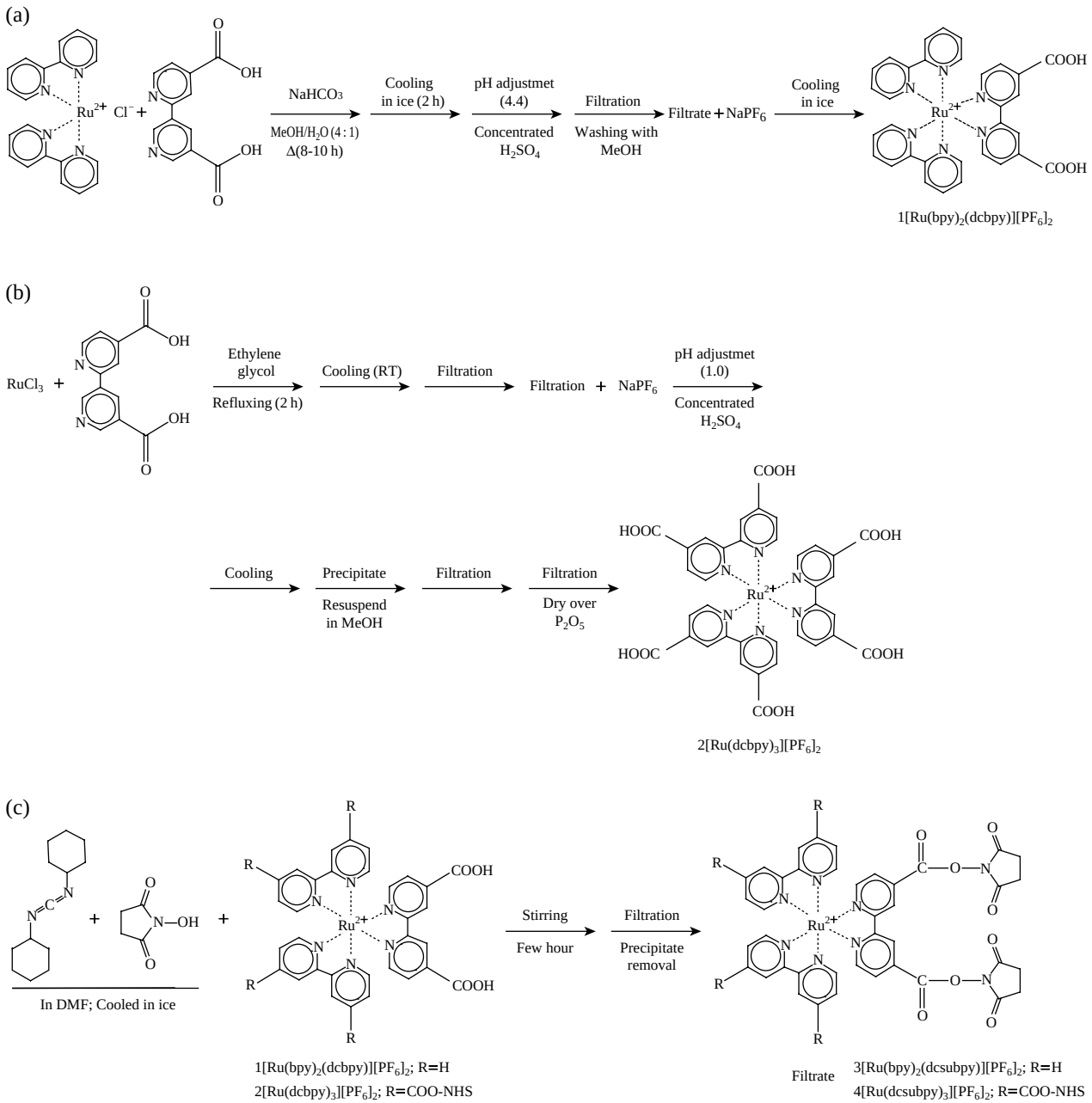

Figure 2. (a) Schematic presentation of the synthesis of Ru bis(2,2′-bipyridine)(2,2′-bipyridine-4,4′-dicarboxylic acid) bis(hexafluorophosphate) (1), (b) Schematic presentation of the synthesis of Ru tris(2,2′-bipyridine-4,4′-dicarboxylic acid)bis(hexafluorophosphate) (2), (c) Schematic presentation of the synthesis of N-hydroxysuccinamide (NHS) esters (3) and (4).

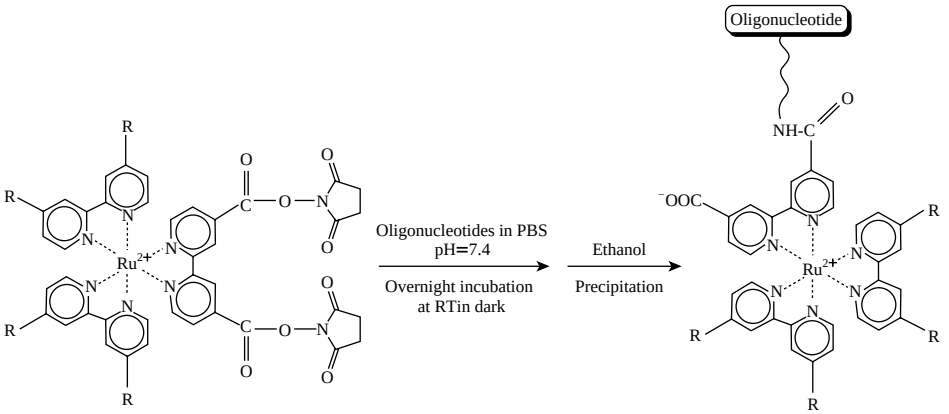

Figure 3. Schematic presentation of the labeling of oligonucleotides with N-hydroxysuccinamide (NHS) esters (3) and (4).

nium (II) ($[Ru(bpy)_3]^{2+}$) display an emission from charge-transfer states with decay times ranging from 100 to 4,000 ns in fluid solutions with reasonable quantum yields[20]. In addition, complexes of ruthenium, osmium, and rhenium[21-23] have the advantages of being usable over a range of absorption and emission wavelengths, as well as a range of decay times. We synthesized a less symmetrical Ru-complex, $[Ru(bpy)_2(dcbpy)]$[24] and covalently coupled it to oligonucleotides. Synthesis of the ECL-probes and their application to label oligonucleotides are based on previous research[15,24] and are only of academic interest.

Synthesis of Ru bis(2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid) bis(hexafluorophosphate) (1)

The complex 1 was synthesized by refluxing $Ru(bpy)_2C1_2$ with dcbpy in a methanol-water mixture followed by acidification. The reaction steps are schematically shown in Figure 2a.

Synthesis of Ru tris(2,2′-bipyridine-4,4′-dicarboxylic acid)bis(hexafluorophosphate) (2)[25]

A symmetrical $[Ru(dcbpy)_3]$ complex was synthesized from $RuCl_3$ by incorporating the dcbpy ligand. Figure 2b shows the reaction schematics.

Synthesis of the N-hydroxysuccinamide (NHS) esters (3) and (4)

Ru tris(2,2′-bipyridine-4,4′-dicarboxylic acid) N-hydroxysuccinimide ester (4) was prepared by allowing the complex 2 to react with a mixture of N,N′-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide in DMF (Figure 2c). The filtrate containing the active Ru-complex was used for labeling the oligonucleotides. The NHS-ester (3) was prepared analogous to (4).

Labeling Oligonucleotides

(NHS) ester (3) or (4) was introduced via the amino group of the modified oligonucleotides. The oligonucleotides were incubated with NHS ester (3) or (4) dissolved in dimethyl sulfoxide overnight at room temperature in the dark (Figure 3). The oligonucleotides recovered from these labeling reactions by ethanol precipitation were further purified by reversed-phase HPLC.

Structure and Fabrication of Microelectrode Array

The microelectrode array was designed so that it can, in an embodiment, be integrated with a photodiode array chip allowing on-chip handling of solutions and ECL detection. To simplify the assembly of the microelectrodes, we examined several different electrode configurations, including a coplanar interdigitated cathode and anode. The basic arrangement attempts of the electrodes are shown in Figure 4. Since the anode area determines the ultimate detection limit, the integrated electrode should provide more surface area but without significantly encroaching more real estate. Therefore, different geometrical patterns were tried to maximize the anode surface area. A coplanar, wave-shaped configuration of the anode electrodes was found to be the best arrangement. However, in the cases of comb-shaped and wave-shaped anode arrangements, as the distance between two electrodes (cathode and anode) increases, the anode surface area decreases, and the uniformity of the ECL intensity lessens. Therefore, the coplanar, closely-placed cath-

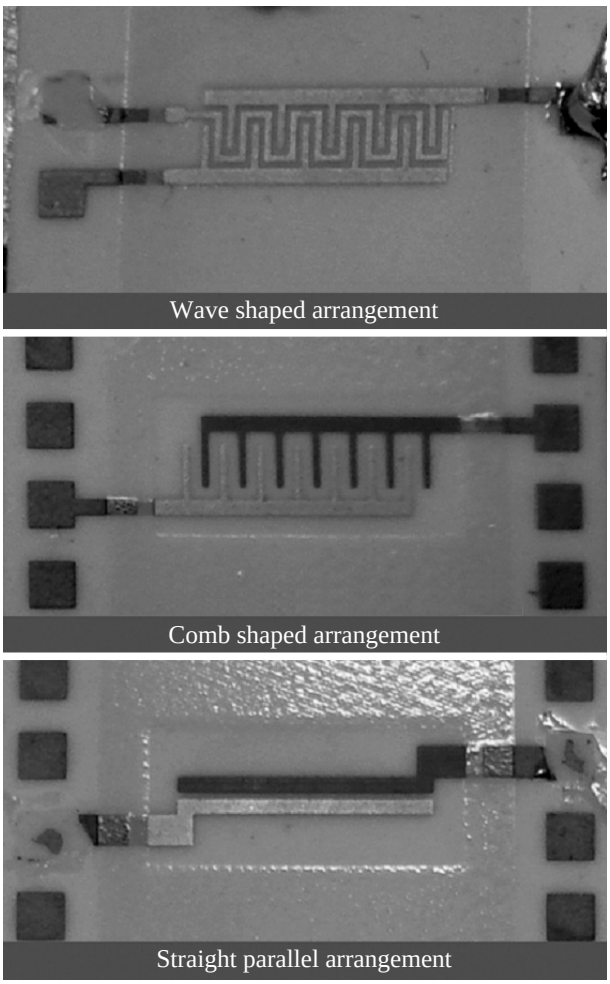

Figure 4. Representative photo images of the microelectrodes with different electrode configurations.

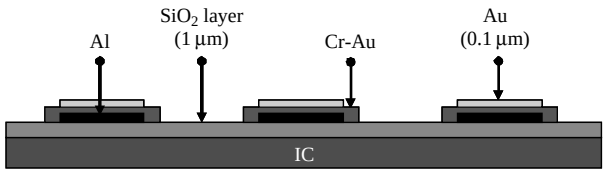

Figure 5. Exploded view of microelectrode array showing three substrates: integrated circuit (IC), SiO_2 passivation layer, and gold sputtered aluminum.

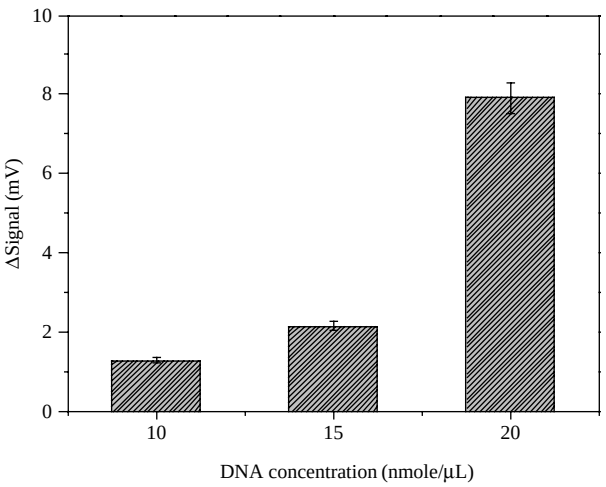

Figure 6. Recorded signal intensity (mV) vs. calculated mol of a single strand. The minimum detectable quantity was found to be 10 nmol/μL.

ode and wave-shaped anode has been, to date, the best electrode arrangement for the microelectrode array in terms of sensitivity and linearity (data not shown).

The ECL microelectrode construction is shown in the exploded view of Figure 5. The entire system is created by the vertical assembly of an integrated circuit, silicon substrate, and metal electrodes. An array of aluminum (Al) thin film was first deposited selectively onto the silicon substrate (passivation layer). The SiO_2 layer thickness is 1 μm. The Al film was then sputtered with a thin gold layer (0.1 μm) with a 0.1-0.21 μm Cr adhesion layer.

Determination of DNA Based on the ECL Detection

A detection limit of 50 nmole of TBR with a sample volume of 5 μL, i.e., 10 nmole/μL was recorded using a straight parallel electrode configuration where the distance between two adjacent electrodes was 24 μm (signal/noise ≥ 3) (data not shown). A silicon photodiode diode coupled with a digital oscilloscope operated at room temperature was used for signal detection.

The recorded signal of ECL light intensity, given in mV versus Ru (II) chelate labeled DNA loading is plotted in Figure 6. A detection limit of 10 nmol DNA is obtained (signal/noise ≥ 3). This detection limit is poor compared to that of the commercial QPCR 5000 (Perkin Elmer) which claims a 10 amol detection limit using a cooled PMT and photon counting techniques. However, with further development of the detection photodiode detection elements and signal amplification techniques we expect to achieve a better detection limit.

Conclusions

A microelectrode array successfully measured ECL

and detected ECL-probe labeled single-stranded DNA with a lower detection limit of 1 nmole with a sample volume as low as 1 μL. It is worth mentioning that ECL detection is not limited in application to DNA quantification. As a versatile, highly sensitive, chemical analysis tool it can be conveniently applied to other chemical sensing and metrology applications. The microelectrode array is thus destined for eventual use in an embodiment wherein the microelectrode array will be integrated with a semiconductor photodiode array to allow for ECL-based on-chip detection and quantification of biomolecules.

Materials and Methods

$RuCl_3$, $Ru(bpy)_2C1_2$ and $Ru(bpy)_3C1_2$, N,N′-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide, sodium hexafluoro phosphate ($NaPF_6$), methanol, ethanol, ethylene glycol, dimethyl formamide (DMF), tripropylamine (TPA) and other chemical reagents were purchased from Sigma (St. Louis, MO), and used as received without further purification. Oligonucleotides with sequence 5′-NH_2-CTT CCT CTG TAG CTT GCT CT-3 were purchased from GeneChem Inc. (Daejeon, South Korea). This sequence was chosen as it represents the conserve region of an HIV gag gene. Water (>18.0 MΩ), purified using a Millipore Nanopure water system, was first autoclaved and then used for all experiments.

The NHS-ester of $[Ru(bpy)_2(dcbpy)]^{2+}$ and of the more symmetric complex $[Ru(dcbpy)_3]^{2+}$ were synthesized following the methods described previously[24].

Synthesis of Ru bis(2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid) bis(hexafluorophosphate) (1)

$Ru(bpy)_2C1_2$ (0.4 g), $NaHCO_3$ (0.4 g), and 2,2′-bipyridine-4,4′-dicarboxylic acid (0.3 g) were heated in MeOH : H_2O=4 : 1 for 8-10 h. After cooling the solution in an ice bath for 2 h, the pH was adjusted with concentrated H_2SO_4 to 4.4. The formed precipitate was filtered and washed with MeOH. The filtrate was treated with 5 g $NaPF_6$ in 25 mL H_2O, cooled in an ice bath, and the precipitate was collected by filtration.

Synthesis of Ru tris(2,2′-bipyridine-4,4′-dicarboxylic acid)bis(hexafluorophosphate) (2)

$RuCl_3$ (0.1 g), 2,2-bipyridine-4,4′-dicarboxylic acid (3.67 g) were suspended in ethylene glycol (15 mL), and refluxed for 2 h. The solution was cooled down to room temperature and filtered. The filtrate was treated with 2.5 g $NaPF_6$ in 25 mL H_2O, the pH was adjusted to 1.0 with concentrated H_2SO_4 and cooled in an ice bath for a few hours, and the precipitate was collected by filtration. The precipitate was resuspended in MeOH, filtered and dried over P_4O_{10}.

Synthesis of Ru tris (2,2′-bipyridine-4,4′-dicarboxylic acid) N-hydroxysuccinamide (NHS) ester (4)

DCC (0.46 g) and 0.238 g of N-hydroxysuccinimide were dissolved in 3 mL of DMF with stirring and cooled in an ice bath. A solution of 0.38 g of Ru tris (2,2′-bipyridine-4,4′-dicarboxylic acid) (2) was then added, and the mixture was stirred for a few hours. The formed precipitate was filtered, and the filtrate containing the active Ru-complex was used for labeling the oligonucelotides. The NHS-ester (3) was prepared similarly.

Labeling Oligonucleotides

Ru-NHS esters were introduced via the amino group of the modified oligonucleotides. The oligonucleotides (0.1 μmol) in 100 μL of phosphate-buffered saline (pH 7.4) were reacted with 5 μmol of Ru-NHS ester dissolved in dimethyl sulfoxide (400 μL) overnight at room temperature in the dark. The oligonucleotides were recovered from the labeling reactions by precipitation with ethanol. The labeled oligonucleotides were further purified by reversed-phase HPLC on a Vydac C18 semiprep column with mobile phases of reagent A (tetraethylammonium acetate, 100 mmol /L, pH 7.0) and reagent B (equal volumes of reagent A and acetonitrile), running the gradient from 20% to 40% B.

ECL experiments were performed on the microelectrode with free Ru-complex and with Ru-chelate labeled DNA. Samples were spotted on the electrodes directly. The chemiluminescence produced by the reaction of the oxidized TPA and Ru-chelate depends on the concentration of both chemicals. In this work, the concentration of TPA was kept constant (50 mM) and the metal chelate concentration or melatl chelate-labeled DNA concentration was varied. The solutions were prepared as follows: The Ru-complex was dissolved in 50 mM TPA and a phosphate buffer (pH 7.5) to make a 10 mM stock solution of the metal complex. The stock solution was then diluted with a 50 mM TPA solution to produce a set of test solutions, with varying metal-chelate concentrations ranging from 1 nM to 5 mM.

The electrode voltage was controlled by a potentiostat. The emitted light was detected by a silicon photodiode. The output was fed through an electronic arrangement as described in our previous work[6-9] and

recorded with a digital oscilloscope and computer.

Acknowledgements

This research was sponsored by the Korean Ministry of Commerce, Industry and Energy under the contract 10023590-0000-00 program with Seoul National University.

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