Published in Biosensors and Bioelectronics 22, issue 5, 722-729, 2006
which should be used for any reference to this work

Electrochemiluminescent hybridization chip with electric field aided mismatch discrimination

Anna-Maria Spehar-Deleze [a,b,*], Laurette Schmidt [c], Reinhard Neier [c], Sakari Kulmala [b], Nico de Rooij [a], Milena Koudelka-Hep [a]

[a] *Laboratory of Sensors, Actuators and Microsystems, Institute of Microtechnology, University of Neuchâtel, Rue Jaquet-Droz 1, CH-2007 Neuchâtel, Switzerland*
[b] *Laboratory of Inorganic and Analytical Chemistry, Helsinki University of Technology, P.O. Box 6100, FIN-02015 HUT, Finland*
[c] *Laboratory of Organic Chemistry, University of Neuchâtel, P.O. Box 2, CH-2007 Neuchâtel, Switzerland*

Abstract

This paper describes a heterogeneous DNA-hybridization assay based on electrochemiluminescence (ECL) detection on gold electrodes. Short, 15-mer oligonucleotides were conjugated with a synthesized electrochemiluminescent label, bis(2,2′-bipyridine)-5-isothiocyanato-1,10-phenanthroline ruthenium(II) at the amino-modified 5′-end. Gold electrodes were derivatized with 15-mer oligonucleotide probes via 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS) cross-linking reaction and hybridized with Ru-labeled strands. Two types of self-assembled-monolayers have been utilized for the immobilization reaction, 3-mercaptopropanoic acid (3-MHA) and 16-mercaptohexadecanoic acid (16-MHA). Longer thiols were more stable at high electrode potentials needed for the ECL generation. The system was sensitive down to 1 fmol of labeled complementary strand, detected in 30 μL of buffer. Mismatch discrimination was achieved both passively by washing and actively by application of negative electrode potential on electrodes prior to detection, but active denaturing lead to better results. Two base-pair mismatches were discriminated at room temperature.

Keywords: Electrochemiluminescence; Immobilization; Oligonucleotide; Electric field; Mismatch discrimination

1. Introduction

The progress of Human Genome project in the past decade has lead to the expansion of the field of molecular diagnostics and brought DNA-analysis into many scientific fields, including pathology, genetics, medical laboratories and drug discovery. Different DNA microarray based technologies and DNA biosensors have been developed to fulfill the demands for faster, simpler and cheaper analytical methods. Specific gene sequences can be analyzed by direct sequencing or DNA-hybridization, latter being more commonly used due to its simplicity and possibility for high-throughput analysis. Most DNA-hybridization detection methods currently rely on fluorescence imaging (Benoit et al., 2001; Dharmadi and Gonzales, 2004), but other methods, such as electrochemical (Wang, 2000; Gasparac et al., 2004; Wong et al., 2005), microgravimetrical (Su et al., 2004), bioluminescence (Kricka, 1999), chemiluminescence (Cheek et al., 2001) and electrochemiluminescence (ECL) (Miao and Bard, 2003; Spehar et al., 2004; Bertolino et al., 2005; Firrao, 2005) methods have been used.

ECL is a general term used to describe a reaction or mechanism, which produces light at the surface of an electrode. ECL generation methods and applications are regularly reviewed (Knight, 1999; Fahnrich et al., 2001; Richter, 2004). The most common ECL luminophore is tris(2,2′-bipyridine)ruthenium(II) ($Ru(bpy)_3^{2+}$), due to its ability to produce ECL at room temperature, in an aqueous solution and in the presence of numerous compounds, usually denoted as coreactants (Knight, 1999). The ECL of $Ru(bpy)_3^{2+}$ has been used for detection amino acids and peptides (Wang and Bobbitt, 1999; Hendrickson et al., 2000), codeine (Michel et al., 1999) and DNA (Dennany et al., 2003), to name just a few applications.

$Ru(bpy)_3^{2+}$ is commonly used as an electrochemiluminescent label in immunoassays and in DNA-assays, often in

* Corresponding author. Tel.: +41 793783119; fax: +41 327205711.
E-mail address: anna-maria.spehar@hut.fi (A.-M. Spehar-Deleze).

combination with polymerase chain reaction (PCR) (Blackburn et al., 1991; Boom et al., 1999; Bruno and Kiel, 1999; de Jong et al., 2000; Zhang et al., 2001). Ruthenium labeled antibodies and PCR amplified DNA strands are typically immobilized on the surface of paramagnetic microbeads, which are then magnetically captured on the surface of an electrode. Then ECL signal is generated in the presence of *n*-tripropylamine (TPA), which is the most efficient known coreactant (Leland and Powell, 1990). While $Ru(bpy)_3^{2+}$ is by far the most commonly used ruthenium complex in ECL-based analytical applications, other ruthenium complexes, such as $Ru(phen)_3^{2+}$ is also known to exhibit ECL of comparable or higher intensity (Michel et al., 1999; Arora et al., 2001; Kuwabara et al., 2003). A ruthenium derivative having two bipyridine and one phenanthroline ligand used for codeine determination was observed to generate 2.5-times higher intensity of ECL (Michel et al., 1999).

Electrochemical detection methods are of great interest in DNA-analysis due to simple instrumentation, high sensitivity, low-cost and compatibility with microfabrication techniques (Wang, 2003; Lucarelli et al., 2004). An obvious advantage of ECL with respect to fluorescence is that no excitation source is needed as the light generating reaction is triggered electrochemically. In addition, it shares characteristics of electrochemical methods, such as highly localized reaction and spacial control. However, only a few examples of heterogeneous ECL-based hybridization assays have been reported (Miao and Bard, 2003; Bertolino et al., 2005). Miao and Bard (2003) performed an ECL assay on gold using 23-mer oligonucleotides. Bertolino et al. (2005) fabricated a silicon based ECL chip with interdigitated gold electrodes and integrated photodiode. The system was able to discriminate 25% mismatched strands. Firrao (2005) detected $Ru(bpy)_3^{2+}$-labeled DNA-strands on glassy carbon electrodes, obtaining detection limit of 10 pmol for the hybridized complementary strand. Amino-modified probes were immobilized on carbon via by aid C–N bond, formed by the aid of an applied electric field. This immobilization method is very simple and does not require surface treatment; however, as the immobilization can occur also through secondary amines present in a DNA strand, control of the surface coverage and orientation of DNA probe molecules is complicated. In addition, microfabrication of glassy carbon is still at early stage of development compared with noble metals.

Immobilization of the DNA strands on gold can be accomplished by incorporation of a functional mercapto group on oligonucleotide during the synthesis. However, this process is very tedious because the procedure for the modification of DNA is complicated, and the yield of synthesis of mercapto-containing DNA is quite low (Ge et al., 2003; Huang et al., 2000). Another common way to immobilize biomolecules on gold is via mercapto-carboxylic acids, which in the presence of carbodiimides can form amide bond with an amino group present in a biomolecule. The most common carbodiimide is 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), which is widely used for conjugation of biological substances. It catalysis formation of amide bonds between carboxylic acids or phosphates and amines by activating carboxyl or phosphate group to form an *O*-acylisourea derivative, which then rapidly reacts with primary amines (Hermanson, 1996). Covalent immobilization of amino-modified oligonucleotides and proteins is achieved this way. *N*-Hydroxysuccinimide (NHS) is frequently added to the EDC solution, resulting in formation of an intermediate active ester, which then reacts with amine. Thus, the final product is the same, but the intermediate is more stable and therefore the reaction yield is usually higher (Hermanson, 1996).

Theoretical calculations have shown that the electrostatic surface effects influence immobilization and hybridization kinetics of DNA strands, as well as stability of the formed duplexes (Vainrub and Pettitt, 2003). In practice it has been shown that, for example, hybridization kinetics of long DNA strands (157–864 bases) with short probes immobilized on indium–tin-oxide electrodes was considerably faster upon application of low voltage of 200 mV between the electrodes (Su et al., 2002). Positive potential of +300 mV has been reported to increase immobilization kinetics of thiolated oligonucleotides on gold electrodes (Peterson et al., 2001; Heaton et al., 2001; Swami et al., 2005), while negative potential of −300 mV was reported to cause denaturing of hybridized mismatched duplexes while leaving complementary duplexes intact (Heaton et al., 2001). Common to above-mentioned examples is that the applied electric field is so low, that only non-charging, faradaic currents are induced and the electrodes are maintained withing the ideally polarizable region. In an approach described by Sosnowski et al. (1997), electric current pulses were applied on hybridized strands. Single base mismatch discrimination was achieved in DNA duplexes over length of 6–27 nucleotides. In order to protect DNA from unwanted electrochemical side reactions electric current might induce, such as local pH change and possible radical formation, the immobilization was performed on 1-μm thick agaroze gel layer previously deposited on the electrodes.

The aim of this research was to develop an ECL-based hybridization chip and to investigate effect of an applied electrode potential on mismatch discrimination. Chip consisted of gold electrodes was microfabricated and derivatized as described below. Ruthenium labeled oligonucleotides were used as ECL luminophores for hybridization detection. Mismatch discrimination experiments were performed by application of low electrode potential.

2. Experimental

2.1. Chemicals and materials

A set of complementary 15-mer oligonucleotides having C_6 linker and amino-modification at the 5′-end were purchased from MicroSynth, Switzerland. A strand having sequence 5′-NH_2-TTGCTAAGGATCATT-3′ was used as a probe. Complementary target 5′-NH_2-AATGATCCTTAGCAA-3′, and mismatched target 5′-NH_2-AATGAT**TCT**C**G**AGCAA-3′ with mismatched bases indicated in bold text were labeled with ruthenium complex (Section 2.3). Sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate

dihydrate, sodium tetraborate decahydrate, potassium chloride, tris(hydrosymethyl)aminomethane (Tris), dimethylsulfoxide (DMSO), dimethylformamide (DMF), magnesium chloride hexahydrate, sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), glycine (mixture of L- and D-isomers), *n*-tripropylamine (TPA), 3-mercaptopropanoic acid (3-MPA), 16-mercaptohexadecanoic acid (16-MHA), 90%, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 1-methylimidazole, hexaamineruthenium(III) chloride (RuHex), thiophosgene, calcium carbonate and ammonium hexauorophosphat were all acquired from Sigma–Aldrich, Switzerland. Ruthenium trichloride, 99%, 2,2′-bipyridyl, lithium chloride, tin dichloride dihydrate, 5-amino-1,10-phenanthroline were obtained from Acros, Switzerland and ethanol (0.2% H_2O) from Merck, Switzerland. AZ 1518 photoresist was product of Clariant and Dow Corning poly(dimethylsiloxane) (PDMS) kit Sylgard 184 from Distrelec, Switzerland.

2.2. Synthesis of Ru-1 and Ru-2

Bis(2,2′-bipyridine)-5-amino-1,10-phenanthroline ruthenium(II) (Ru-1) was synthesized according to previously published procedures (Sprintschnik et al., 1977; Garcia-Fresnadillo and Orellana, 2001; Youn et al., 1995). Briefly, bipyridine and tri-chlororuthenium were heated and refluxed during 3 h in DMF. After solvent evaporation the resulting mixture was crystalized at 0 °C with acetone, refluxed with water–ethanol solution and treated with lithium chloride. The resulting compound was refluxed with 5-amino-1,10-phenanthroline in the ethanol–water solution during 3 h. After ethanol was evaporated hexauorophosphate ammonium salt was added. The resulting yellow Ru-1 compound was purified in a chromatography column. Finally, the amino group of Ru-1 was converted to the active isothiocyanato group of Ru-2 compound with thiophosgene in the presence of calcium carbonate and dry acetone.

2.3. Ruthenium labeling of oligonucleotides

Oligonucleotides were labeled according to a slightly modified published procedure (Molecular probes, 2003). Briefly, prior to labeling, oligonucleotides were purified by chloroform extraction and precipitated with ethanol. Amino-modified oligonucleotides were then dissolved in 100 mM tetraborate buffer, pH 8.5, to obtain a concentration of approximately 250 μM. Ru-2 was dissolved in a small volume of DMSO and added to the oligonucleotide solution at 30-fold concentration with respect to the oligonucleotides. This mixture was left to react in the dark and was gently shaken during 6 h. Labeled oligonucleotides were precipitated by addition of a 0.1:2.5 (v/v) of 3 M NaCl: cold, absolute ethanol with respect to the labeling solution. The mixture was kept 30 min at −20 °C and then centrifuged 30 min at 12,000 rpm. The supernatant was removed and the pellet was rinsed twice with cold 70% ethanol. Labeled oligonucleotides were allowed to dry in air during 10 min, and were stored at −20 °C until use.

2.4. Instrumentation and methods

The concentration of the labeled and unlabeled oligonucleotides was determined by UV–vis measurements, performed in 50 mM tetraborate buffer, pH 7.8 using Hewlett-Packard 8453 spectrophotometer. The labeling efficiency was calculated to be 100% using absorption of oligonucleotides at 260 nm and of ruthenium complex at 455 nm. The cyclic voltammograms were recorded using Autolab PGSTAT12 (Eco chemi) potentiostat in a three-electrode mode against a silver pseudoreference electrode on chip made by placing a droplet of silver conductive glue on a gold electrode (Spehar et al., 2004). Cyclic voltammograms were measured in 10 mM tris buffer, pH 7.0 sweeping potential first in negative direction, and back to positive. The ECL potential was generated using PAR 273 potentiostat and the signal was measured with a PMT tube (Hamamatsu H5701-50, Switzerland) through an optical filter with a bandwidth 600 ± 80 nm controlled with a home-written Labview program which collected points at a frequency of 8 Hz. A voltage of −950 V was supplied to the PMT using a laboratory-built high-voltage power supply. ECL measurements were done in a 300 mM phosphate buffer, pH 7.8, containing 100 mM TPA and 0.1% SDS. The ECL signal was generated by steping potential from 0 to 1.15 V for a pulse period of 300 ms and steping back to 0 V. Denaturing experiments using electric field were done in 30 mM phosphate buffer, pH 7.0. All the measurements were performed at room temperature.

2.5. Preparation of oligonucleotide-modified gold electrodes

Gold electrodes were microfabricated by a lift off process on Pyrex wafers using positive, AZ 1518 photoresist. A thin titanium layer of 20 nm was evaporated as a seed layer followed by 100 nm of polycrystalline gold. Then 400-nm thick layer of silicon nitride was deposited by plasma enhanced chemical vapor deposition (PECVD) on the structured electrodes and the electrode surface area was opened by nitride etching. A at PDMS layer was made by mixing 10:1 ratio of a prepolymer and a curing agent. The solution was degassed under vacuum and cured 4 h at 65 °C. A reservoir was made by pinching a hole and the PDMS layer was reversible sealed around electrodes. Fig. 1 shows a ready gold chip with three working electrodes, an integrated silver pseudoreference electrode and a counter electrode. The diameters of the working electrodes are 100, 300 and 500 μm, respectively. While all three working electrodes were used for preliminary experiments and optimization, all results reported in this paper are obtained on the largest electrode. Before immobilization, the electrodes were cleaned in 50% sulfuric acid containing 5% hydrogen peroxide during 10 min, rinsed with copious amounts of water and dried under a stream of nitrogen. Self-assembled-monolayers of 3-MPA and 16-MHA thiols were formed during 1 h from 1 mM solution prepared in absolute ethanol. Upon soaking in thiol solution, the electrodes were cleaned with ethanol and water and dried in a stream of nitrogen. The amino-modified oligonucleotides were immobilized on SAM covered electrodes via

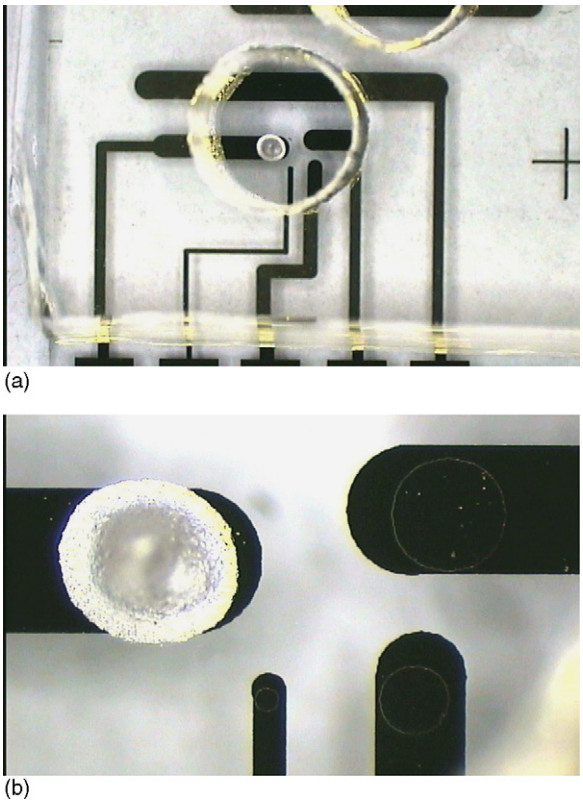

Fig. 1. Au chip used for heterogeneous ECL assay. The volume of the PDMS reservoir is 30 μL. Lower figure is a close up of the electrodes. Diameter of the largest electrode is 500 μm, middle 300 μm and small 100 μm. Silver pseudoreference electrode was made by placing a droplet of silver glue on a gold electrode and curing it at 120 °C during 2 h (left).

EDC/NHS coupling (Hermanson, 1996), which leads to covalent bond between surface carboxylic acid and amino-terminal of the oligonucleotide. The oligonucleotides were dissolved in 100 mM 1-methylimidazole buffer, pH 7, containing 100 mM of $MgCl_2 \cdot 6H_2O$ into which, prior to immobilization, freshly prepared EDC and NHS were added to obtain concentrations of 100 and 75 mM, respectively. Then 10 μL of oligonucleotide solution was carefully pipetted on three working electrodes taking care not to cover reference electrode and left for 3 h in a humid chamber. Non-covalently attached probes were washed away with ethanol and water and the chips were dried in a nitrogen stream.

2.6. Hybridization

Upon probe immobilization the unreacted EDC/NHS groups were deactivated by treating the electrodes with a 30 mM phosphate buffer containing 10 mM glycine and 0.2% SDS during 30 min. Then the surface was blocked by treatment with 35 μg/mL of single stranded calf thymus DNA during 10 min. Labeled complementary and mismatched strands were pipetted on working electrode and allowed to incubate 2 h, unless otherwise indicated. Hybridization was performed in 30 mM phosphate buffer, pH 7.5, containing 100 mM $MgCl_2 \cdot 6H_2O$.

3. Results and discussion

3.1. Optimization of the ECL conditions

ECL is very dependent on pH, applied voltage, presence and type of surfactants. Based on previous experience, we first measured the ECL signal in a 300 mM phosphate buffer containing 100 mM TPA, pH 7.8 (Spehar et al., 2004). However, the intensity of the ECL was very low (Fig. 2a). Therefore, the influence of surfactants was investigated, namely that of cationic surfactant cetyltrimethylammonium bromide (CTAB) and of an anionic surfactant (SDS), respectively. Addition of SDS strongly increased the ECL intensity, with intensity maximum obtained in the presence of 0.1% SDS, further increase of the surfactant concentration causing slight decrease of the signal. CTAB also increased the signal, however, less in comparison to SDS. Therefore, further optimization experiments and the measurements on DNA-modified electrodes were done in the presence of 0.1% SDS.

The optimal potential for the ECL generation was found by performing a CV at 50 mV/s from 0 to 1.3 V. The maximum ECL signal was obtained at 1.15 V versus a silver reference electrode integrated on the chip (Fig. 2b). This potential was subsequently used for the ECL detection on DNA-modified electrodes.

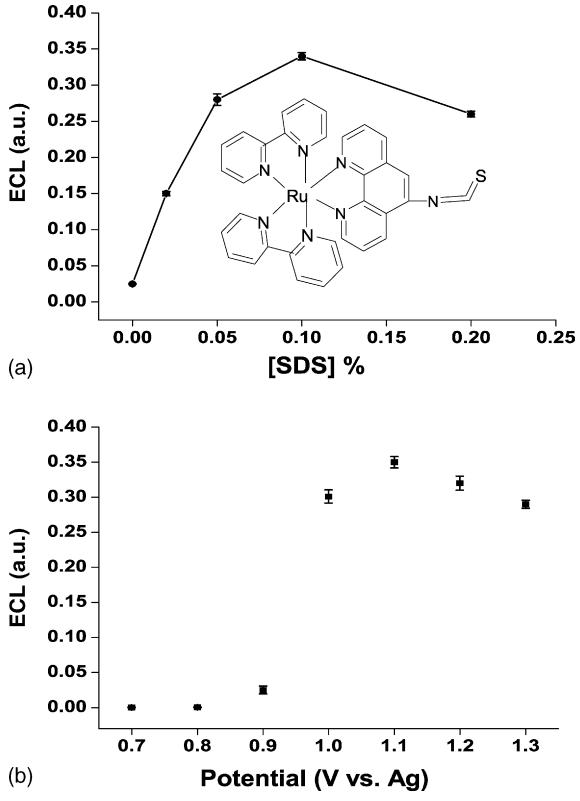

Fig. 2. ECL intensity of 1 μM Ru-1 solution in 300 mM phosphate buffer containing 100 mM TPA, pH 7.8 vs. concentration of SDS (a), potential dependence of ECL generation on a hybridization chip vs. Ag pseudoreference electrode (b) in the presence of 0.1% SDS. Measurements were performed on the 500 μm diameter electrode. The insert in the upper figure shows molecular structure of Ru-2 complex. The experiments were repeated in triplicate and the standard deviation is shown in the error bars.

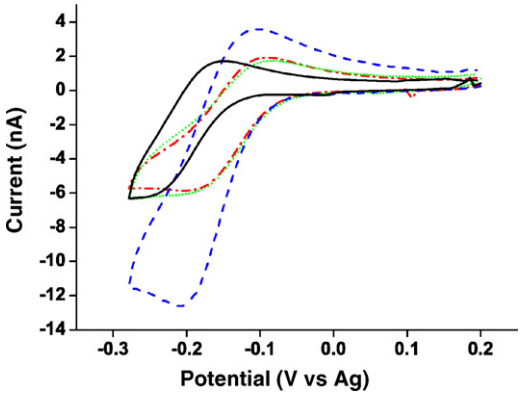

Fig. 3. Cyclic voltammograms of 20 μM RuHex solution in 10 mM tris buffer pH 7.0, obtained at bare gold electrodes (dash–dot–dash curve), at a 16-MHA modified electrode (solid curve), at a 16-MHA/EDC/NHS modified electrode (dotted curve) and at 16-MHA/EDC/NHS/DNA modified electrode, treated with the probe solution of 1 μM (dashed curve). Sweep rate is 50 mV/s.

3.2. *Voltammetric characterization of the modified surface*

Voltammetry is a commonly used method for characterization of modified electrodes. We used RuHex ($Ru(NH_3)_6^{3+}$) for surface characterization, as it binds electrostatically to DNA strands due to the negatively charged phosphate backbone and has previously been used for surface coverage determinations of thiolated oligonucleotides on gold (Steel et al., 1998; Yu et al., 2003). The 3-MPA and 16-MHA SAMs were formed on a series of chips in identical conditions (Section 2.5). Fig. 3 shows cyclic voltammograms obtained on unmodified clean gold, 16-MHA thiolated gold, the ester covered gold (EDC/NHS treatment) and DNA-modified gold surface. Voltammograms on two different types of monolayers are very similar, indicating no detectable difference in monolayer formation or probe immobilization efficiency.

It can be seen that voltammograms are very similar on unmodified and on ester covered gold (EDC/NHS treatment), while the reduction peak on SAM-covered surface is less pronounced and both oxidation and reduction peaks are shifted. The immobilization of DNA strands clearly increased the size of the reduction and oxidation peaks of CV. This is due to the accumulation of RuHex at the electrode surface upon binding to the oligonucleotide strands. It can be noticed that the reduction peak is considerably more pronounced compared to the oxidation peak. Previously, RuHex voltammograms have been shown on similarly prepared surface, where the reduction peak of RuHex decreased upon DNA immobilization (Huang et al., 2000). A probable explanation for this difference in our results is that we immobilized DNA strands with their 5′-end, and thus expect them to be in an upright position, whereas in the other case amino groups present in a DNA strand were used to achieve immobilization, resulting in a longitudinal positioning of DNA with respect to the surface. No difference in peak splitting was observed upon surface treatment, $\Delta E_p \approx 100$ mV, was observed in all cases. To calculate the surface coverage, we integrated right-sided half of the reduction peak of the oligonucleotide-modified surfaces, subtracted area of the voltammograms not containing DNA, multiplied the resulting area by two and calculated probe density using following equations (Yu et al., 2003):

$$\Gamma_{Ru} = \frac{Q}{nFA} \tag{1}$$

$$\Gamma_{DNA} = \Gamma_{Ru} N_A \frac{z}{m} \tag{2}$$

where Γ_{Ru} is the surface saturation of RuHex, Q the charge, n the number of electrons transferred in the reaction, F the Faraday constant, A the electrode surface area, Γ_{DNA} the probe coverage, z the charge of RuHex, m the number of nucleotides in a DNA strand and N_A is the Avogadro constant. The obtained value for the surface coverage upon treatment with 1 μM probe solution is about 9×10^{12} molecules/cm^2 (dashed curve voltammogram in Fig. 3), which is within typical surface coverage range of DNA probes (10^{11}–10^{13} molecules/cm^2) (Gooding, 2002; Steel et al., 1998). Divalent Mg^{2+}-ions were added in both immobilization and hybridization buffers, because their presence produce more densely packed probe films, due to decreased electrostatic repulsion between adjacent DNA strands (Boon et al., 2002). Thus, we are confident that our SAM formation and probe immobilization were successful due to (i) the difference in voltammograms upon surface treatment with mercaptocarboxylic acid and probe solution, (ii) increase in reduction current of RuHex upon surface treatment with increasing concentrations of probe solution and (iii) the reductive current becomes constant at probe concentration of 600 nM and higher. This indicates saturation of available probe binding sites.

3.3. *ECL on DNA-modified electrodes*

Fig. 4 shows results of ECL assays, obtained on a series of chips modified with probe solutions of different concentrations (10, 100 pM, 1, 10, 50, 100, 500 nM and 1 μM) and incubated with a constant concentration (1 μM) of labeled complementary

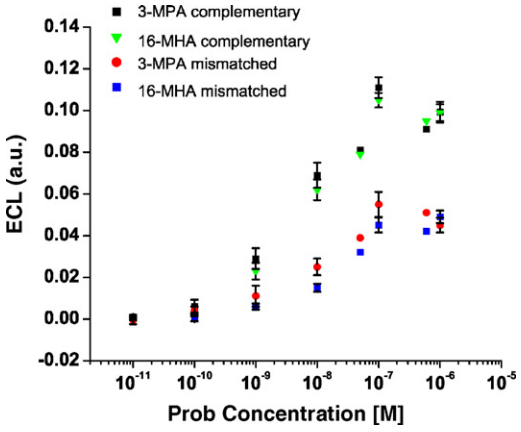

Fig. 4. ECL calibration curves obtained upon hybridization of constant concentration of Ru-2-labeled complementary and mismatched target on 3-MPA/EDC/NHS and 16-MHA/EDC/NHS monolayers. *Conditions*: ECL was generated by steping potential from 0 to 1.15 V vs. Ag pseudoreference for a pulse period of 300 ms. Signal was recorded using PMT through an optical filter of bandwidth 600 ± 80 nm. Buffer used was 300 mM phosphate, 100 mM TPA and 0.1% SDS, pH 7.8. The experiments were repeated in triplicate and the standard deviation is shown in the error bars.

and mismatched target. SAM/EDC/NHS modified chips served as negative controls. After the incubation time of 2 h, chips were washed with 30 mM phosphate buffer, pH 7.0, containing 0.2% SDS and heated to about 38 °C. Results obtained on 3-MPA and 16-MHA surfaces were found quite similar, but the intensity of the ECL is approximately 10% higher on 3-MPA surface than on 16-MHA surface. This is probably due to the shorter distance between the label moiety and the electrode surface.

The negative controls and the chips derivatized with probe solution of 10 pM resulted in comparable intensity, indicating that non-specific adsorption is very low. Measurable signal was obtained upon electrode derivatization with 10 μL of probe solution of concentration 100 pM, which corresponds to 1 fmol oligonucleotide. It can be seen that in all cases the highest ECL intensity was obtained on chips treated with a probe solution of 100 nM. From that we concluded that at this probe concentration occurs at the optimal surface coverage, which lead to the most efficient hybridization. Peterson et al. (2001) have shown that at higher probe densities hybridization efficiency decreases due to the steric hindrance and electrostatic repulsion caused by the immobilized probes. Signals obtained from mismatched duplexes were considerably lower (approximately 50%) than upon hybridization with the complementary strand.

One very important difference between two types of monolayers was found during the course of the work. Longer thiols were more resistant to high positive potential needed for the ECL generation. A probable explanation is better organization and higher adsorption of longer alkanethiol chains compared to the shorter ones (Rowe and Creager, 1994). The optimal potential for ECL generation in our case was 1.15 V versus Ag pseudoreference integrated on a chip. Gold-thiol bond is unstable at high voltages, so to minimize thiol desorption we used short pulse time of 300 ms for the ECL generation. In these conditions, the oligonucleotides immobilized on 3-MPA surface produced higher intensity ECL, however, signals obtained at 16-MHA monolayer were more repeatable. On 16-MHA monolayer the ECL signals could be measured tens of times, with washings in between without decrease of the signal, demonstrating that the formed monolayer was stable and no desorption of probes or denaturing of the hybrids did occur. However, new monolayers were formed for all new assay experiments for caution. The experiments with electrostatic field denaturing were performed only on 16-MHA formed SAMs.

3.4. Effect of the applied electric field on mismatch discrimination

As could be seen from Fig. 4, mismatch discrimination was achieved by careful surface treatment and electrode washing. However, we were interested if the selectivity could be still improved by control of the electrode potential. The possibility to control surface potential of the immobilization platform and thus the processes occurring on the surface is of particular interest when work is done on electrodes, in contrary to non-conductive surfaces typically used in fluorescence assays.

A set of chips were modified with probe solutions of different concentrations (100 pM, 1, 10, 100 nM and 1 μM) and a constant concentration of complementary and mismatched Ru-labeled strands (1 μM) and allowed to hybridize in passive conditions for 3 h. After washing with denaturing buffer (30 mM phosphate buffer, pH 7.0), the ECL signal of the electrodes was recorded in the ECL buffer. Then, denaturing buffer was placed into the reservoir and negative potential of −300 mV was applied on the electrode during different time period. The ECL signal was always recorded in the ECL buffer.

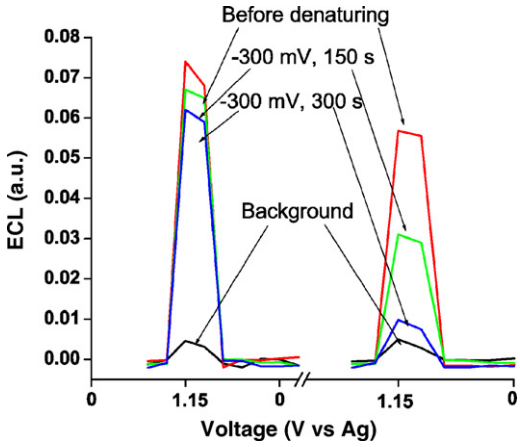

Fig. 5. ECL signal on the electrode modified with 10 nM probe solution and incubated with Ru-labeled complementary (left) and mismatched strand (right) during 3 h, prior and after application of the negative potential of −300 mV vs. Ag-pseudoreference electrode for the indicated time. ECL measurement conditions are as described in Fig. 4, while negative electrode potential was applied in the denaturing buffer (30 mM phosphate, pH 7.0).

Fig. 5 shows typical ECL response upon application of negative potential on the electrodes modified with complementary (a) and mismatched duplexes (b). It can be seen that upon application of the potential of −300 mV for 150 s, the ECL signal of complementary duplex remained unchanged, while the signal of the mismatched strand decreased approximately 50%. Further application of the negative electrode potential decreased slightly the ECL signal of the complementary strand, while the signal from mismatched strand decreased close to the background. Same experiment was performed in the ECL buffer (300 mM phosphate, 100 mM TPA and 0.1% SDS), but no denaturing was observed neither in the case of complementary nor mismatched duplex. The negative electrode potential of −300 mV was used, as no electrochemical side relations, such as electrolysis of water or damage to DNA take place and the electrode is maintained in an ideally polarized region (Heaton et al., 2001; Su et al., 2002). This is very important point, because the distance of the oligonucleotides from the electrode surface is only around 2–3 nm (d(C–C) = 0.14 nm, Sutton, 1965) and electrode potential of −300 mV cause a field gradient on the order of 10^8 V/m. It is known that electrostatic surface conditions do influence stability of a formed duplex, and that the distance up to which surface conditions influence surface bound species depends strongly on ionic strength of the solution (Vainrub and Pettitt, 2003). Thus, application of the negative electrode potential is expected to destabilize the formed duplex, the mismatched more compared to the matched, and the effect is expected to be more pronounced in lower ionic strength solutions. This could explain why we did

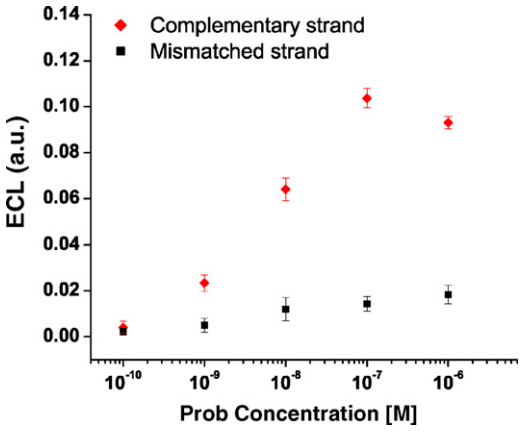

Fig. 6. ECL calibration curves obtained upon hybridization (3 h) of constant concentration of Ru-labeled complementary and mismatched target on 16-MHA/EDC/NHS modified electrodes. ECL measurements were performed after application of negative electrode potential of −300 mV during 300 s. Denaturing using negative potential caused mismatched target to dehybridize while matched hybrid gave high intensity ECL signal. ECL measurement and denaturing conditions were same as in Fig. 5. The experiments were repeated in triplicate and the standard deviation is shown in the error bars.

not observe denaturing effect of the mismatched duplex in the ECL buffer, however, the influence of the surfactant (0.1% SDS) was not investigated and cannot be ruled out.

Fig. 6 presents calibration curve obtained on DNA-modified electrodes using denaturing voltage of −300 mV during 300 s prior to the ECL measurement. These results show that by controlling electrode potential successful discrimination between mismatched and perfect complementary strand can be achieved.

Comparison of Figs. 4 and 6 shows that better mismatch discrimination results were obtained by electric field aided denaturing compared to washing, e.g. for concentration of 10 nM of probe solution, the hybridization result for mismatched strand after washing is around 40%, while after electrostatic denaturing only around 20% of complementary strand. A strong appeal of this approach is relatively simple mismatch discrimination achieved by control of the electrode potential in room temperature compared to extensive washing.

4. Conclusions

Synthesized ruthenium complex exhibited high ECL intensity in the presence of 0.1% anionic surfactant SDS and potential of 1.15 V versus silver pseudoreference electrode. Microfabricated gold electrodes were derivatized with 15-mer, 5′amino-modified oligonucleotides using mercaptocarboxylic acid SAM and EDC/NHS coupling. Two different SAMs, 3-MPA and 16-MHA were studied. The performance in terms of immobilization was very similar, but 16-MHA SAM was more stable. Ru-labeled complementary strands were detected down to a picomolar concentration, corresponding to a total amount of 1 fmol of the immobilized probe. Two base-pair mismatch discrimination was achieved with and without application of an electrode potential; however, mismatched discrimination was considerably better when negative electrode potential of −300 mV was applied during 300 s prior to the ECL detection.

References

Arora, A., Eijkel, J.C.T., Morf, W.E., Manz, A., 2001. Anal. Chem. 73, 3282–3288.

Benoit, V., Steel, A., Torres, M., Yu, Y.-Y., Yang, H., Cooper, J., 2001. Anal. Chem. 73, 2412–2420.

Bertolino, C., MacSweeney, M., Tobin, J., O'Neill, B., Sheehan, M.M., Coluccia, S., Berney, H., 2005. Biosens. Bioelectron. 21, 565–573.

Blackburn, G.F., Shah, H.P., Kenten, J.H., Leland, J., Kamin, R.A., Link, J., Peterman, J., Powell, M.J., Shah, A., Talley, D.B., Tyagi, S.K., Wilkins, E., Wu, T.G., Massey, R.J., 1991. Clin. Chem. 37, 1534–1539.

Boom, R., Sol, C., Gerrits, Y., Boer, M.D., van Dillen, D.P.W., 1999. J. Clin. Microbiol. 37, 1489–2147.

Boon, E.M., Salas, J.E., Barton, J.K., 2002. Nat. Biotech. 20, 282–286.

Bruno, J., Kiel, J.L., 1999. Biosens. Bioelectron. 14, 457–464.

Cheek, B.J., Steel, A.B., Torres, M.P., Yu, Y.-Y., Yang, H., 2001. Anal. Chem. 73, 5777–5783.

de Jong, M.D., Weel, J.F.L., Schuurman, T., Wertheim-van Dillen, P.M.E., Boom, R., 2000. J. Clin. Microbiol. 38, 2568–2573.

Dennany, L., Foster, J., Rusling, J., 2003. J. Am. Chem. Soc. 125, 5123–5128.

Dharmadi, Y., Gonzales, R., 2004. Biotechnol. Prog. 20, 1309–1324.

Fahnrich, K.A., Pravda, M., Guilbault, G.C., 2001. Talanta 54, 531–559.

Firrao, G., 2005. Int. J. Env. Anal. Chem. 85, 609–612.

Garcia-Fresnadillo, D., Orellana, G., 2001. Helv. Chim. Acta 84, 2708–2730.

Gasparac, R., Taft, B.J., Lapierre-Delvin, M.-A., Lazareck, A.D., Xu, J.M., Kelley, S.O., 2004. J. Am. Chem. Soc. 126, 12270–12271.

Ge, C., Liao, J., Yu, W., Gu, N., 2003. Biosens. Bioelectron. 18, 53–58.

Gooding, J.J., 2002. Electroanalysis 14, 1149–1156.

Heaton, R.J., Peterson, A.W., Georgiadis, R.M., 2001. Proc. Natl. Acad. Sci. 98, 3701–3704.

Hendrickson, H.P., Anderson, P., Wang, X., Pittman, Z., Bobbitt, D.R., 2000. Micromech. J. 65, 189–195.

Hermanson, G.T., 1996. Bioconjugate Techniques. Academic press, San Diego, CA.

Huang, E., Zhou, F., Deng, L., 2000. Langmuir 16, 3272–3280.

Knight, A., 1999. Trends Anal. Chem. 18 (1), 47–62.

Kricka, L.J., 1999. Clin. Chem. 45, 453–458.

Kuwabara, T., Noda, T., Ohtake, H., Toyama, S., Ikariyama, Y., 2003. Anal. Biochem. 314, 30–37.

Leland, J.K., Powell, M.J., 1990. J. Electrochem. Soc. 137, 3127–3131.

Lucarelli, F., Marrazza, G., Turner, A.F., Mascini, M., 2004. Biosens. Bioelectron. 19, 515–530.

Miao, W., Bard, A.J., 2003. Anal. Chem. 75, 5825–5834.

Michel, P.E., Fiaccabrino, G.C., de Rooij, N.F., Koudelka-Hep, M., 1999. Anal. Chim. Acta 392 (2–3), 95–103.

Peterson, A.W., Heaton, R.J., Georgiadis, R.M., 2001. Nucleic Acids Res. 29, 5163–5168.

Molecular probes, M., 2003. Amine-reactive Probes. Molecular probes, Eugene, USA, MP00143.

Richter, M., 2004. Chem. Rev. 104 (6), 3003–3036.

Rowe, G.K., Creager, S.E., 1994. Langmuir 10, 1186–1192.

Sosnowski, R.G., Tu, E., Butler, W.F., O'Connell, J.P., Heller, M.J., 1997. Proc. Natl. Acad. Sci. 94, 1119–1123.

Spehar, A.-M., Koster, S., Kulmala, S., Verpoorte, E., de Rooij, N., Koudelka-Hep, M., 2004. Luminescence 19, 287–295.

Sprintschnik, G., Sprintschnik, H.W., Kirsch, P.P., Whitten, D.G., 1977. J. Am. Chem. Soc. 99, 4947–4954.

Steel, A.B., Herne, T.M., Tarlov, M.J., 1998. Anal. Chem. 70, 4670–4677.

Su, H.-J., Surrey, S., McKenzie, S.E., Fortina, P., Graves, D.J., 2002. Electrophoresis 23, 1551–1557.

Sutton, L.E., 1956-. Table of Interatomic Distances and Configuration in Molecules and Ions, Supplement 1956–1959, Special publication No. 18. Chem. Soc, London, UK.

Su, X., Robelek, R., Wu, Y.J., Wang, G.Y., Knoll, W., 2004. Anal. Chem. 76, 489–494.

Swami, N.S., Chou, C.-F., Terbergueggen, R., 2005. Langmuir 21, 1937–1941.

Vainrub, A., Pettitt, B.M., 2003. Biopolymers 68, 265–270.
Wang, J., 2000. Electroanalysis 13, 635–638.
Wang, J., 2003. Anal. Chim. Acta 500, 247–257.
Wang, X., Bobbitt, D.R., 1999. Anal. Chim. Acta 383, 213–220.
Wong, E.L.S., Mearns, F.J., Gooding, J.J., 2005. Sens. Actuators B 111–112, 515–521.
Youn, H., Terpetschnig, E., Szmacinski, H., Lakowitz, J.R., 1995. Anal. Biochem. 232, 24–30.
Yu, H.-Z., Luo, C.-Y., Sankar, C.G., Sen, D., 2003. Anal. Chem. 75, 3902–3907.
Zhang, L.T., Schwartz, G., O'Donnel, M., Harrison, R.K., 2001. Anal. Biochem. 293, 31–37.