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Heterogeneous oligonucleotide-hybridization assay based on hot electron-induced electrochemiluminescence of a rhodamine label at oxide-coated aluminum and silicon electrodes

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Abstract

This paper describes a heterogeneous oligonucleotide-hybridization assay based on hot electron-induced electrochemiluminescence (HECL) of a rhodamine label. Thin oxide-film coated aluminum and silicon electrodes were modified with an aminosilane layer and derivatized with short, 15-mer oligonucleotides via diisothiocyanate coupling. Target oligonucleotides were conjugated with tetramethylrhodamine (TAMRA) dye at their amino modified 5′ end and hybridization was detected using HECL of TAMRA. Preliminary results indicate sensitivity down to picomolar level and low nonspecific adsorption. The sensitivity was better on oxide-coated silicon compared to oxide-coated aluminum electrodes and two-base pair mismatched hybrids were successfully discriminated. The experimental results presented here might be useful for the design of disposable electrochemiluminescent DNA biosensors.

Keywords: Electrochemiluminescence; Hot electron; Rhodamine; Immobilization; DNA

1. Introduction

DNA specific interactions, such as hybridization, interaction with drugs and carcinogens, and DNA damage are subjects of wide scientific interest for which new methods allowing more efficient detection are constantly investigated. Of particular interest is the detection of DNA strands of specific sequence due to applications in forensic science, food analysis, pharmacology, genomics, etc. Most DNA hybridization assays rely on fluorescence detection, but other methods, such as quartz crystal microbalance [1], electrochemistry [2,3], chemiluminescence [4] and anodic electrochemiluminescence (ECL) [5–9] have also been applied.

ECL is a method, where light is generated on the surface of an electrode. In anodic ECL, an electrochemiluminescent luminophore (usually tris(bipyridine)ruthenium, $Ru(bpy)_3^{2+}$) is oxidized on a surface of a metal or carbon electrode and light is generated upon reaction with a coreactant (typically *n*-tripropylamine) [5–9].

In hot electron-induced ECL (HECL) light generation process is initiated by a tunnel emission of hot, energetic electrons from a thin insulator film-covered electrode into an electrolyte solution upon cathodic polarization. Many common photoluminescent and chemiluminescent luminophores have been detected this way, examples including luminol and its derivatives [10,11], $Ru(bpy)_3^{2+}$ [12,13], fluorescein and eosin [14], SYBR (R) Green I [15], coumarine dyes [16], Rhodamine B [17] and terbium chelates [18,19]. The most sensitive HECL luminophores so far are terbium labels, which can be detected down to femtomolar concentration [18,19]. An obvious advantage of both anodic and hot electron-induced ECL with respect to fluorescence is that no excitation source is needed as the light generating reaction is triggered by electrode polarization. A distinct advantage of HECL over anodic ECL is that various luminophores with different optical and redox properties can be simultaneously excited [14,20].

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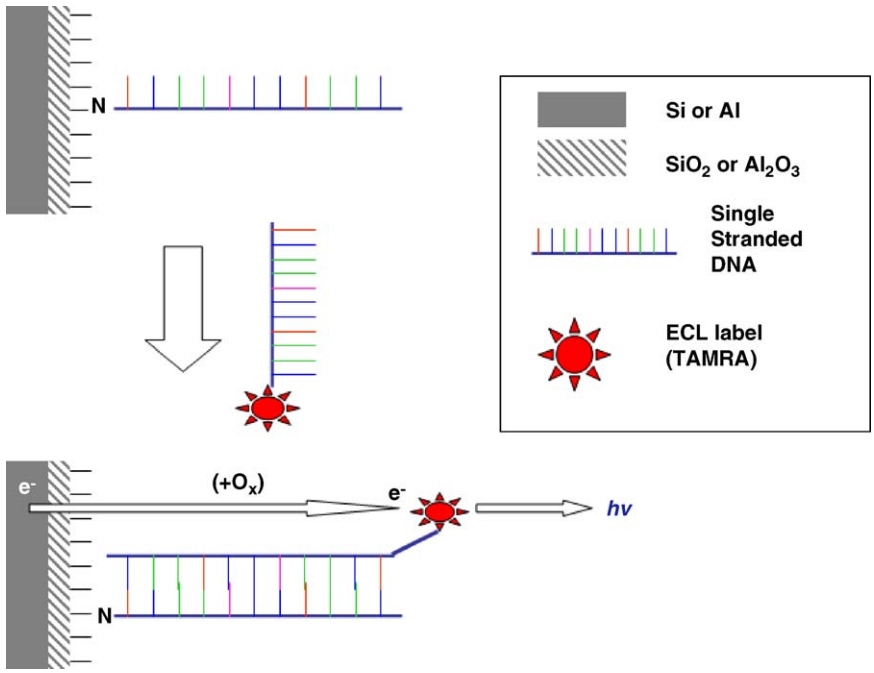

Fig. 1. A scheme representing hybridization assay on amino-modified electrode surface and HECL based hybridization detection. Figure not to scale.

HECL has been previously used for biorecognition detection in immunoassays [12,21–23]. Capturing antibodies were physically adsorbed on oxide-coated aluminum [12,21,22] and silicon [23] electrodes and the biorecognition reaction was detected using target antibodies labeled with HECL labels. Immunoassays and nucleic acid probing techniques share many similarities, as they are both based on a unique biorecognition process, however, there are some fundamental differences due to the fact that antibodies are relatively large molecules (MW $\approx$ 160 kDa) while DNA-assays utilize short oligonucleotides (MW $\approx$ 5–10 kDa). In addition, antibodies contain numerous amino and carboxylic acid groups and can be readily physically adsorbed on unmodified oxide surfaces by combination of electrostatic and hydrophobic interactions. On the other hand, immobilization of oligonucleotides requires addition of a functional group during synthesis that allows their coupling to a surface in a controlled manner, most typical modifications being amino and thiol groups. A surface used for immobilization of oligonucleotides must be flat, homogeneous, thermally and chemically stable, and a reproducible surface treatment that allows high density of DNA strands and offers low background must be feasible. In addition, to achieve massively parallel assays, compatibility with microfabrication technology is required.

Most common approach for immobilization of oligonucleotides is introduction of an amino-functionality onto a solid support. Self-assembled aminosilane films have been deposited on a wide variety of hydroxylated surfaces, including glass [24–26], silicon dioxide [27–29], silicon nitride [30], indium-tin-oxide [31] and aluminum oxide [32,33]. The requirement for silane deposition is the presence of OH-groups on the surface, which allows formation of Si–O bond between surface oxygen and silicon in the silanization reagent.

In this article, we present a novel method for the detection of DNA hybridization based on HECL of tetramethylrhodamine (TAMRA). TAMRA is widely used label in fluorescence [34] and fluorescence resonance energy transfer based assays [35]. We used TAMRA-labeled target DNA strands as the HECL luminophores, because it is a commercially available labeling reagent, and our recent study has shown that parent compound of TAMRA, Rhodamine B, can be detected down to subnanomolar level using HECL [17]. Fig. 1 shows a scheme of HECL hybridization detection on a thin oxide film-coated Al- or Si-electrode.

2. Experimental

2.1. Chemicals and materials

Oligonucleotides were synthesized and HPLC-purified by Microsynth, Switzerland (Table 1). Target oligonucleotides were labeled in laboratory at their amino-modified 5′ end (Section 2.3) and mismatched bases are indicated in bold font. Sodium tetraborate decahydrate, sodium azide, sodium nitrate, potassium phosphate buffer, pH 7.5, ethanol (<0.2% H_2O) and

Table 1
Structure of the used oligonucleotides

Oligonucleotide	Sequence	Name
Probe	5′-NH_2-TTGCTAAGGATCATT-3′	P
Complementary target	5′-NH_2-AATGATCCTTAGCAA-3′	TAMRA-CT
Mismatched target	5′-NH_2-AATGAT**T**CT**G**AGCAA-3′	TAMRA-MT
Noncomplementary target	5′-NH_2-AAAAAAAAAAAAAAA-3′	TAMRA-NT

methanol (hypergrade for liquid chromatography) were obtained from Merck. Pyridine, aminopropyltriethoxysilane (APTES), *N*-(2-aminoethyl)-3-aminopropyl-trimethoxysilane (EDA), 1,4-phenylene diisothiocyanate (PDC), L-glycine, sodium dodecyl sulfate (SDS), *N*,*N*-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were acquired from Sigma–Aldrich. Succinimidyl ester of 5-(and-6)-carboxytetramethyl-rhodamine (TAMRA) was product of Molecular Probes. For HECL-measurements, boron-doped *p*-Si (1 0 0) with resistivity of 0.01–0.02 Ω cm (Okmetic Oy, Finland) and aluminum electrodes from 99.9% pure aluminum band, 0.3 mm thick (Merck Art. 1057, batch 720 K22720857) were used.

2.2. Instrumentation and methods

UV–vis absorbance spectra were measured with Hewlett-Packard 8453 spectrophotometer using 1-cm optical pathlength quartz cuvette. HECL measurements were carried out by using single photon counting with an instrumentation that consisted of a Perkin-Elmer MP 1993 photon counting module with yellow sensitive cathode and Nucleus MCS-II scaler card, similar to previously described system [17]. The HECL signal was filtered with an optical filter of bandwidth 550 ± 40 nm. The cell used consisted of a sample holder made of Teflon, a fine Pt-wire counter electrode and a disposable silicon or aluminum working electrode. The effective area of the electrode in this cell was 63.6 mm^2. A laboratory-made coulostatic pulse generator [22] was applied to generate cathodic pulses and a pulse generator was adjusted to yield cathodic pulses with 40 μC of charge and −40 V of voltage with frequency of 20 Hz. Contact angles were measured with drop shape analysis system (Kruss). Aluminum electrodes were coated with 2–3 nm thick natural oxide film, while silicon electrodes were thermally oxidized in clean room to yield 4 nm thick silicon dioxide layers. Briefly, the wafers were cleaned using standard RCA-cleaning process with HF-dip last. The oxidation was performed at 850 °C in 10% oxygen and 90% nitrogen atmosphere immediately upon cleaning. The thickness of the silicon dioxide layer was determined using He–Ne ellipsometer operated at 632.8 nm and refraction index of the SiO_2 fixed at 1.465. The oxidation and oxide thickness monitoring processes are described in more detail elsewhere [13]. Silicon electrodes were diced to 9 mm × 9 mm and aluminum electrodes cut to 10 mm × 10 mm pieces. Absorbance measurements were performed in 0.05 mol/L sodium tetraborate buffer at pH 7.8 which was adjusted with 1 M sulfuric acid, and HECL measurement were performed in the same buffer into which prior to measurement sodium azide was added as coreactant in concentration of 0.01 M.

2.3. TAMRA labeling of oligonucleotides

Oligonucleotides were labeled according to slightly modified published procedure [36]. Briefly, prior to labeling, oligonucleotides were purified by chloroform extraction and precipitated with ethanol. Then amino-modified oligonucleotides were dissolved in 0.1 M tetraborate buffer, pH 8.5, to obtain concentration of 250 μM. This solution was then added to a small amount of TAMRA–DMSO solution, so that the dye concentration was approximately 30-fold to the concentration of the oligonucleotide. The mixture was left to react in the dark and was gently shaken during 6 h. Labeled oligonucleotides were precipitated twice 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. Preparation of oligonucleotide-modified electrodes

Prior to silanization, aluminum and silicon electrodes were cleaned with methanol and water and dried in a nitrogen flow. Liquid phase aminosilanization was performed according to slightly modified procedures [24,30] under ambient conditions using freshly made APTES-EDA solution (mixing ratio 1:1) at 5% total silane concentration (v/v) in methanol/water solution (99.5:0.5, v/v) for 1 h. The substrates were sequentially washed with methanol and water, dried in a nitrogen stream and cured in oven at 115 °C for 1 h. The surface was activated in a freshly prepared solution of distilled DMF and pyridine (9:1, v/v) containing 1 mM PDC during 2 h at room temperature. The oligonucleotides were then immobilized by placing a droplet of 20 μL of amino-modified oligonucleotides in a 40 mM phosphate buffer, pH 7.0, containing 0.150 M sodium nitrate (sodium nitrate was used instead of more commonly used sodium chloride, because chloride ions can have detrimental effect on very thin oxide films) on the surface and left overnight in a humid chamber. The substrates were sequentially washed with methanol and water, then soaked into 0.01 M glycine solution prepared in 40 mM phosphate buffer, pH 7.0, for 30 min to deactivate surface isothiocyanate groups, and dried in a nitrogen stream.

2.5. Hybridization

Labeled targets were dissolved in the same buffer as used for immobilization, 30 μL of this solution was carefully pipetted in the center of an electrode and left to incubate 3 h at ambient temperature. The electrodes were then washed with tetraborate buffer, pH 7.8, into which 0.2% SDS was added, heated to 35 °C, dried in a nitrogen stream and stored in dessicator until HECL measurement.

3. Results and discussion

3.1. UV–vis properties of the labeled compounds

The concentration of the oligonucleotides and labeling efficiency were determined by UV–vis spectroscopy. Fig. 2 shows UV–vis spectra of Rhodamine B solution (a), unlabeled 15-mer oligonucletide (b) and TAMRA-labeled 15-mer oligonucleotide (c) measured in 0.05 M tetraborate buffer, pH 7.8.

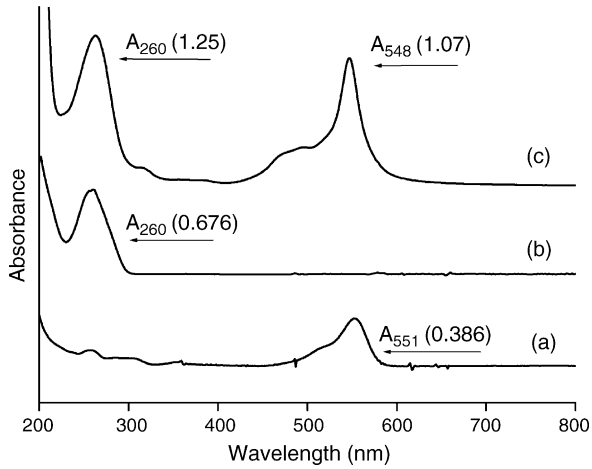

Fig. 2. Absorbance spectra of Rhodamine B (a), unlabeled probe (b) and TAMRA-labeled target (c). Measured in 0.05 M tetraborate buffer, pH 7.8, using 1-cm optical pathlength quartz cuvette.

The ratio of TAMRA to oligonucleotide is 1.4, calculated from the absorbance peaks at 260 for oligonucleotide using absorption coefficient 155,000 $cm^{-1}\,L^{-1}$ mol (given by provider) and 548 nm for TAMRA using absorption coefficient 95,000 $cm^{-1}\,L^{-1}$ mol [34]. No effort was made to further separate labeled oligonucleotides from nonspecifically associated dye.

3.2. HECL of TAMRA-labeled DNA on aluminum and silicon oxide-coated electrodes

Prior to hybridization experiments, HECL intensity of TAMRA-labeled DNA was investigated. Aluminum electrodes covered with native, 2–3 nm thick oxide layer [33] and *p*-type silicon electrodes coated with 4 nm thick, thermally grown oxide layer were used in this study [13]. Fig. 3 shows calibration curves obtained on oxide-film coated aluminum and silicon electrodes in the presence of 0.01 M NaN_3 as coreactant [17]. Azide ions were used as coreactants, because previous study has shown that they quench background electroluminescence while slightly enhancing HECL intensity of Rhodamine B [17]. Upon cathodic pulse polarization of oxide-coated aluminum or silicon electrode, hot electrons tunnel through the oxide film into the electrolyte solution generating background ECL. Several paralel mechanims contribute to generation of this background, but it can be mostly explained by F^+-center (electron trapped in an oxygen vacancy) luminescence at the oxide/electrolyte interface solid state high-field electroluminescence inside the oxide film [37]. In addition, it has been suggested anion vacancies are formed at the surface of the electrode partially in response to the oxidizing action of the electrode surface [37]. From Fig. 3, it can be seen that the sensitivity is better on silicon electrodes compared to aluminum, and TAMRA-labeled DNA could be detected down to concentration of 5×10^{-11} mol/L. Background electroluminescence was lower on silicon compared to aluminum electrodes, which is in agreement with previous results [13] (also Section 3.4).

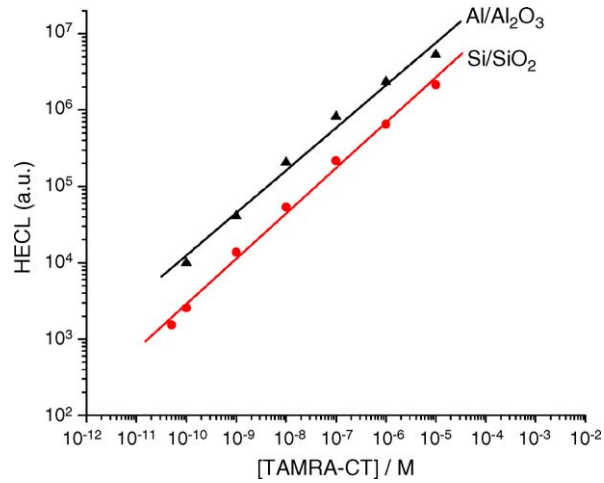

Fig. 3. Calibration curve of TAMRA-labeled oligonucleotides obtained on *p*-silicon electrodes coated with 4 nm thick oxide layer and on aluminum electrodes coated with natural aluminum oxide. *Conditions*: 0.05 M tetraborate buffer containing 0.01 M sodium azide, pH 7.8. Pulse voltage, −40 V; pulse charge, 40 μC; pulse frequency, 20 Hz. The HECL intensities were integrated over 1000 excitation cycles and the signal was recorded through an emission filter of bandwidth 550 ± 40 nm.

3.3. Characterization of the modified surfaces

To investigate deposition of the aminosilane layer, contact angle of water on silicon and aluminum was routinely measured upon each step of the surface treatment. A contact angle is the angle at the interface of a drop of pure water and a planar surface, and it provides a measure of hydrophobicity: steeper the angle, greater is hydrophobicity. Table 2 lists the results of contact angle measurement obtained after each preparation step.

As it can be seen from Table 2, the PDC treatment lead to the increase of hydrophobicity. This was expected, as PDC treatment introduces a hydrophobic phenyl ring on the surface [25,26]. The increase of contact angle by 14° upon PDC treatment indicates successful surface modification.

3.4. HECL on the monolayer coated electrodes

The intensity of HECL is quite independent of the pH of solution, being rather constant at pH range 3–10 [13,17], but depends strongly on the thickness of the insulating layer. Highest HECL intensity on silicon and aluminum electrodes is typically observed at oxide thickness 2–5 nm [13]. Thus, a thick silane layer could quench the HECL signal due to the increased thickness of the barrier. We could not measure the thickness

Table 2
Static contact angle on silicon and aluminum electrodes upon surface treatment

Sample	Contact angle
Aminosilanized Si	49 ± 1
PDC activated Si	63 ± 2
Aminosilanized Al	48 ± 3
PDC activated Al	64 ± 3

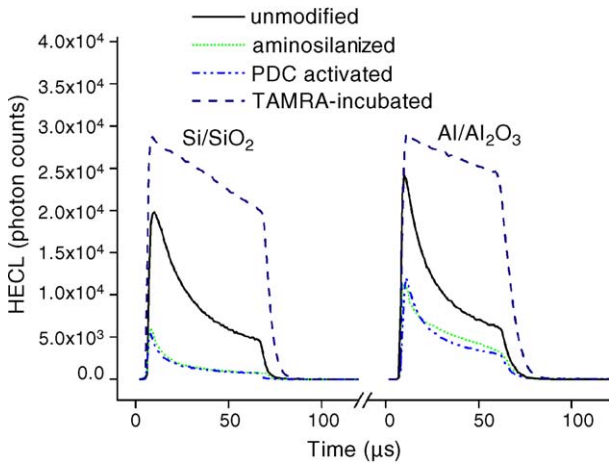

Fig. 4. HECL intensity as a function of time obtained on oxide-coated silicon and aluminum electrodes on: unmodified electrode (solid curve), aminosilane modified electrode (short dot curve), PDC activated electrode (dash–dot curve) and modified with 1 μM solution of TAMRA-label (dash curve). Experimental conditions as in Fig. 3.

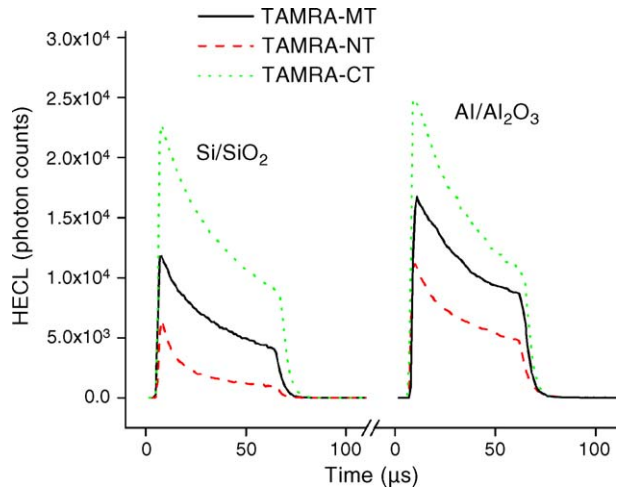

Fig. 5. HECL intensity as a function of time obtained on oxide-coated aminosilane/PDC modified silicon and aluminum electrodes derivatized with 500 nM probe solution and incubated with 1 μM solution of: TAMRA-CT (dot curve), TAMRA-MT (solid curve) and TAMRA-NT (dash curve). Immobilization and hybridization were performed in 40 mM phosphate buffer containing 0.150 M $NaNO_3$, pH 7.0, HECL measurement conditions as in Fig. 3.

of the aminosilane layer, however, based on published reports on similar modification conditions it could be estimated to be around 1 nm on silicon dioxide [25,27], and around 2 nm [32] on aluminum oxide. Another important question is how does the surface treatment influence background electroluminescence, which arises from the reaction of hot electrons with the electrolyte (Section 3.2). If hot electrons would react by luminescent pathways with a deposited monolayer or phenyl groups introduced on the surface, background electroluminescence would increase. To investigate these questions, HECL intensities were recorded on modified and unmodified electrodes (Fig. 4). In addition, we were concerned whether we managed to generate an amino-terminated surface suitable for the oligonucleotide immobilization. Contact angle measurements indicate formation of the silane layer and introduction of phenyl group upon PDC treatment, which indicates amino-terminated surface. To further investigate this question, aminosilanized electrodes were incubated with 1 μM solution of TAMRA labeling reagent dissolved in borate buffer for 3 h. Fig. 4 shows that surface treatment decreased background electroluminescence compared to the untreated electrode, while immobilized label yielded high intensity HECL signal. The difference between modified and unmodified silicon pieces is higher compared to aluminum pieces, and was also very reproducible.

These results (i) confirm successful surface treatment, (ii) demonstrate that used reagents do not react with hot electrons and do not cause increase of the background electroluminescence and (iii) demonstrate that the deposited silane layer does not quench analytical signal. Both HECL signal and background electroluminescence measured on oxide-coated aluminum electrodes are higher compared to oxide-coated silicon, which is in agreement with previous HECL results [13]. It should be pointed out that the injection of hot electrons damages both the oxide and aminosilane layers covering the electrode, and thus this method is suitable mainly for single-use applications.

3.5. HECL on the DNA-modified electrodes

Next, the validity of the method was evaluated by hybridization experiments. Fig. 5 shows HECL intensity as a function of time measured on oxide-coated aminosilane/PDC derivatized silicon and aluminum electrodes modified with 500 nM of probe oligonucleotide and incubated with 1 μM of labeled complementary strand (TAMRA-CT), labeled mismatched strand (TAMRA-MT) and labeled noncomplementary strand (TAMRA-NT). One of the major challenges in DNA analysis is nonspecific adsorption. Noncomplementary DNA strands can adsorb onto the ssDNA-modified surfaces, thus generating background and decreasing sensitivity of the hybridization detection. From Fig. 5, it can be seen the incubation with noncomplementary labeled strand generated signal comparable to background electroluminescence, which is significantly lower on silicon than on aluminum electrodes. This result indicates low nonspecific adsorption and proves that the analytical signal is due to hybridization. While we at present cannot explain reasons for higher background on aluminum compared to silicon electrodes, the most probable explanation is inhomogeneouty and presence of impurities in native aluminum oxide. The signal rise time is different on Al- and Si-electrodes, 10 and 8 μs, respectively.

To further evaluate the validity of presented method for detection of DNA hybridization, hybridization was performed with a DNA-strand containing two mismatched nucleotides (TAMRA-MT). Efficient discrimination of mismatched strands is one of the most powerful tests to evaluate validity of a given method. The mismatched strand having two noncomplementary and 13 complementary bases was efficiently discriminated; the signal is significantly lower than upon hybridization with the complementary strand.

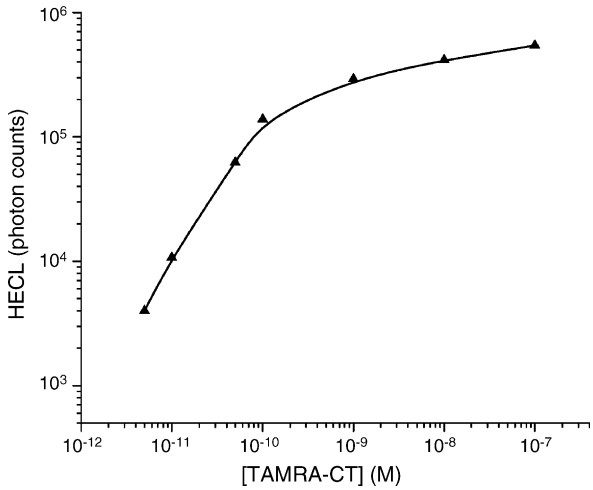

Fig. 6. Calibration plot of TAMRA-CT on oxide-coated aminosilane/PDC modified silicon electrodes derivatized with 500 nM probe solution and incubated with incresing concentrations of target (TAMRA-CT). Immobilization, hybridization and HECL measurement conditions as in Fig. 5.

Finally, an attempt was made to investigate the sensitivity of the system. Oxide-coated aminosilane/PDC derivatized silicon electrodes were modified with a constant concentration of probe solution (500 nM) and incubated with increasing concentration of TAMRA-labeled complementary strand (TAMRA-CT). Fig. 6 shows the obtained results. It can be seen that the system is sensitive down to picomolar concentration of the complementary target. Comparison of the Figs. 3 and 6 shows that the sensitivity of the HECL detection of TAMRA-CT is higher in the heterogeneous assay format than in the solution. There are two explanations for this observation: (i) decrease of the background electroluminescence upon surface modification; (ii) in heterogeneous assay format, the whole sample is concentrated at the surface of the electrode, where the detection occurs.

Two slopes can be observed on the calibration curve; higher steep up to a target concentration of 1×10^{-10} M and lower steep up to a concentration of 1×10^{-7} M. It should be noted that HECL intensity did not increase upon further increase of the target concentration, which indicates saturation of the binding sites. At present we cannot explain reason for non-linear behavior of the calibration curve, but a possible explanation is a change of hybridization kinetics at higher target concentration, due to the electrostatic repulsion of negative phosphate backbone of DNA. Further investigations are necessary to answer this question. Quantification of the immobilized probes was not performed at this stage.

The results presented herein demonstrate potential of HECL for DNA hybridization detection. A strong appeal of this method is the possibility to do multiplexing analysis using wavelength and lifetime discrimination [14,20]. In addition, microfabrication technology for silicon is very developed, and large-scale production of silicon based sensors is readily envisaged. We believe that this method is potentially useful for development of disposable DNA biosensors. Further investigations are in progress in our laboratory.

4. Conclusions

The goal of this work was to investigate potential of HECL detection for heterogeneous DNA hybridization assays. The influence of each preparation step on background and analytical signal was investigated by contact angle measurement and HECL. Our results demonstrate that aminoterminated silane films were successfully deposited on both silicon and aluminum electrodes. It was demonstrated that surface treatment decrease background electroluminescence caused by contact of hot electrons with the electrolyte solution while preserving high analytical signal of HECL luminophore. Nonspecific adsorption was low and two-base pair mismatch was successfully discriminated. Thus, HECL appears to be potentially useful detection method for DNA hybridization. Possible application could include disposable silicon-based DNA-biosensor.

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