Bioelectrochemistry 87 (2012) 78–83

Contents lists available at SciVerse ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Electrochemical detection of DNA hybridization using metallacarborane unit

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ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 26 August 2011
Accepted 28 October 2011
Available online 4 November 2011

Keywords:
Electroanalytical methods
Biosensors
Nucleic acids
Redox labels

ABSTRACT

The evaluation of novel electrochemically active label for electrochemical detection of DNA hybridization is presented. Metallacarborane units modified with iron, cobalt or chromium were investigated. The value of redox potential and relatively strong current signal facilitate usage of Fe-carborane as marker covalently attached to the ssDNA. In electrochemical genosensor the sequence complementary to UL55 gene was labeled and used as a target for biosensor device. Interactions were investigated using electrochemical and piezoelectric methods. Obtained results confirm usefulness of the designed label in electrochemical detection of DNA hybridization.

1. Introduction

The detection of specific sequences of deoxyribonucleic acid (DNA) is of great importance in medicine, biology, food industry and environmental protection [1–4]. One of the method is immobilization of single-stranded DNA fragments onto a solid support and sensing the effects of possible hybridization between these capture probes and analyzed DNA strands [5]. The hybridization event is subsequently detected using either label-free or label-based method [6]. Label (marker, tag) is responsible for the transduction of a biochemical signal (e.g. hybridization in the case of DNA sensing) into a useful analytical signal. Label-free assays are typically simpler, cheaper and less time-consuming than methods employing markers. Moreover, the need for labeling can sometimes change the properties of capture probes, influencing its affinity to the analyte sequence [7]. On the other hand, label-based methods benefit from high sensitivity, resulting from signal enhancement. Thus, the development of ultrasensitive assays, enabling the detection of a particular DNA sequence in extremely small samples, is possible [8]. Typically, a label is covalently attached either to capture probes forming the receptor layer or to target DNA sequence. Following the hybridization, certain physicochemical properties of a label change, providing the analytical signal. Alternatively, marker can be added to the analyte solution and interact with DNA strands via electrostatic, groove or intercalative binding [9].

Electrochemical techniques have gained great attention due to their relatively low cost, simplicity, sensitivity and ease of miniaturization. By coupling the biorecognition elements to the electrochemical transducers, high selectivity can be also achieved. Ferrocene is the most frequently used covalently attached electrochemical marker for DNA sensors [10,11]. Although other metallocenes with different central metals are available, none of them were used until now as DNA-electrochemical label due to air and/or moisture sensitivity [12]. As it was recently reported, also sensors based on ferrocene suffer from certain drawbacks, such as poor electrochemical stability, short storage period and insufficient sensor-to-sensor reproducibility [7]. The performance of ferrocene in complex samples, such as blood serum, was also hampered, probably due to nonspecific adsorption of serum proteins [13]. Ferrocene itself shows limited stability in the phosphoramidite synthesis of oligonucleotide [14]. Among other electroactive tags, attached covalently to DNA strands, osmium tetroxide complexes [15], Redmond Red [16], anthraquinone and Nile Blue [17] should be mentioned. One of the most commonly used alternative to ferrocene, described to date, are metallacarboranes, which enable covalent linkage to oligonucleotide strand for electrochemical detection of DNA [18–20].

Metallacarboranes are a vast family of metallocene-type complexes consisting of at least one carborane cage and one or more metal cations. They are stable, colored crystalline solids that are soluble in organic solvents and well survive exposure to air. Carborane clusters are versatile and efficient ligands for cations of the following metals: Co, Fe, Ni, Cr, Re, Al, Au, Cu, Ir, Mn, Pt etc. This allows for incorporation of variety of metals with different properties into nucleic

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doi:10.1016/j.bioelechem.2011.10.005

acids and its components. An additional advantage of metallacarboranes is susceptibility to redox potential tuning via particular derivatization of the boron cluster ligands [21,22].

Here, the electrochemical detection of DNA hybridization using metallacarborane unit covalently linked to oligonucleotide strand is presented.

2. Experimental

2.1. Apparatus

Electrochemical measurements were conducted with a CHI 660A electrochemical workstation (CH Instruments, USA). Voltammetric experiments were carried out with a three-electrode system consisting of gold disc working electrode (CH Instruments, USA; 2 mm diameter, 3.14 mm^2 surface area), gold auxiliary electrode and Ag/AgCl/1.0 M KCl reference electrode (Mineral, Poland). All potentials are reported versus Ag/AgCl reference electrode at room temperature. To restore the pure gold electrode surface, the electrode polishing kit with alumina powder of different grain sizes was used. The electrochemical solutions were deoxygenated with argon for approximately 15 min prior to data acquisition and were blanketed under an argon atmosphere during the entire experimental period. If not stated otherwise, the cyclic voltammetry (CV) was conducted at a sweep rate of 100 $mV \cdot s^{-1}$, while the square wave voltammetry (SWV) was conducted at a pulse amplitude of 50 mV, increment of 1 mV and a frequency of 10 Hz.

The QCM-D measurements were conducted with Q-Sense E4 instrument and the AT-cut quartz crystal with a fundamental resonant frequency of 4.95 MHz and crystal constant (C) of 17.7 $ng \cdot cm^{-2}$ Hz^{-1} (Q-Sense, Sweden). The effective area of gold sensor exposed to solution is 0.78 cm^2 with roughness less than 3 nm. Such assays allow to measure the change in frequency and energy dissipation which, with the use of a Voigt based representation [23], can be converted into the mass deposited on the gold surface.

2.2. Reagents

Reagent-grade H_2O_2, H_2SO_4, KCl, K_2HPO_4, KH_2PO_4, NaCl, NaOH, HCl, Tris–HCl, DMF (N,N-dimethylformamide), TEAC (tetraethylammonium chloride), MCH (6-mercapto-1-hexanol) were purchased from Aldrich Chemicals. Absolute ethanol, H_2O_2, and H_2SO_4 were received from POCh, Poland. All reagents were used without further purification. All solutions were prepared using Milli-Q water or DMF. Milli-Q water and all aqueous buffer solutions were sterilized using an autoclave.

The metallacarborane/2′-deoxyadenosine conjugate containing iron and cobalt were obtained according to the original procedure described recently [20,24,25]. Briefly, first the 5′- and 3′-hydroxyl functions of 2′-deoxyadonosine were protected using tert-butyldimethylsilyl protection. In the second step, protected 2′′-deoxyguadenosine was activated with 2.5 molar excess of sodium hydride and then treated with a 1.5 molar excess of 8-dioxane-(3,3′-metal-1,21′,2′dicarbollide) adduct [8-O(CH_2CH_2)2O-1,2-$C_2B_9H_{10}$-3,3′-metal-1′,2′-$C_2B_9H_{11}$]. In the third step, the tert-butyldimethylsilyl protective groups were removed with tetrabutylammonium fluoride, providing the metallacarborane/nucleoside conjugate 6-N-{5-[(3,3′-metal-1,2,1′2′dicalbollide-8-yl]3-oxapentoxy}-2′-deoxyadenosine).

Synthesis of 6-N-{5-[(3,3′-iron-1,2,1′2′dicalbollide-8-yl]3-oxapentoxy}-2′-deoxyadenosine 3′-O-(H-phosphonate) was performed by a two-step procedure as described in Ref. [25]. First, the 5′-hydroxyl function of 6-N-{5-[(3,3′iron-1,2,1′2 ′dicalbollide-8-yl] 3-oxapentoxy}-2′-deoxyadenosine was protected with dimethoxytrityl group under standard conditions [26]. Next, the 5′-protected conjugate was transformed into the corresponding 3′-O-(H-phosphonate) according to the PCl_3/triazole method [27].

A sequence complementary to a fragment of the UL55 gene of human cytomegalovirus (HCMV) was selected as a model oligonucleotide. The modified oligomer containing the 3,3′-iron-1,2,1′,2′-dicarbollide at the 5′ end, ^{BEFeC}ACGAGTCTTCGGCTCCGGA-3′, was prepared by solid-phase synthesis (Fig. 1) [28]. Briefly, the unmodified part of the oligonucleotide was synthesized using phosphoramidite chemistry, a β-cyanoethyl cycle and an automated DNA synthesizer [29]. Manual step and H-phosphonate chemistry were used to add the modified monomer bearing the (3,3′-iron-1,2,1′,2′-dicarbollide)-8-yl group [25]. The deprotected metalated oligonucleotide was characterized by HPLC, UV, MALDI-MS and PAGE analysis.

The base sequences were as follows:

thiolated DNA probe:
P1: 5′-TCCGAAGCCGAAGACTCGT-3′-$(CH_2)_6$-SH
target deoxyoligonucleotide modified with metallacarborane:
A1: 5′-^{Fe}ACGAGTCTTCGGCTTCGGA-3′

Two 19-mer deoxyoligonucleotides were purchased from Genomed Sp. z o. o., Poland. The base sequences were as follows:

thiolated DNA probe:
P2: SH-$(CH_2)_6$-5′-TCCGAAGCCGAAGACTCGT-3′
target deoxyoligonucleotide without modification, complementary to P1 and P2:
A2: 5′-ACGAGTCTTCGGCTTCGGA-3′
target deoxyoligonucleotide without modification uncomplementary either to P1 or P2:
A3: 5′-CCCCGTCTCGGAGTGTTGGA-3′

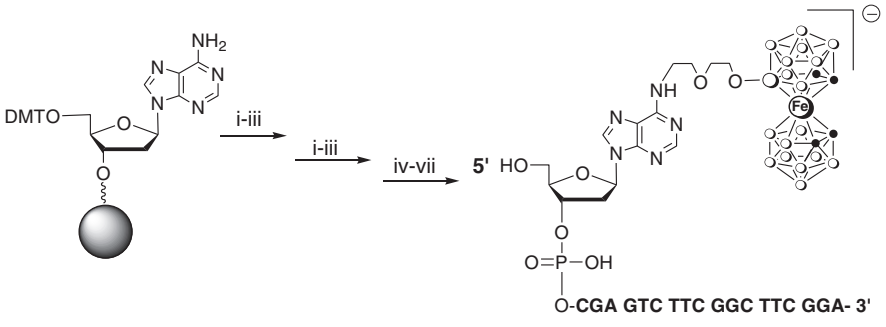

Fig. 1. Synthesis of the oligonucleotide modified with Fe-carborane. i. Detritilation: 3% DCA in CH_2Cl_2; ii. Coupling: unmodified monomers, phosphoramidite chemistry; iii. Oxidation: 0.1 M I_2 in THF/pyridine/H_2O (13:6:1), and capping: 1 M$(CH_3CO)_2O$ in THF/pyridine (1:8) and 0.5 M DMAP in THF; iv. Coupling: modified monomer, H-phosphonate chemistry; v. Oxidation: 10% H_2O in CCl_4, triethylamine, N-methylimidazole (9.0:0.5:0.5); vi. Cleavage from the support: 30% NH_3 aq., 1 h, rt; vii. Base deprotection: 30% NH_3 aq., 2 h, 50 °C.

All oligonucleotide stock solutions were prepared with 10 mM Tris–HCl, (pH 7.5) and stored in a −20 °C freezer before use.

2.3. Solutions

The following solutions were prepared: piranha solution (H_2O_2: H_2SO_4; 3:1); base piranha solution (H_2O_2: H_2O: NH_4OH; 1:5:1); "immobilization buffer" solution, IB, containing 1 M KH_2PO_4 (pH 4.5); "hybridization buffer" solution, HB, containing 10 mM Tris–HCl and 1 M NaCl (pH 7.0). The electrochemical markers and their conjugates with adenosine were investigated in DMF solution of 0.1 M TEAC. Buffer solution for electrochemical measurements of prepared biosensors was composed of 0.05 M K_2HPO_4/KH_2PO_4 and 0.3 M NaCl (pH 7.0). The pH was adjusted with either NaOH or HCl solution.

2.4. Methods

The electrochemical properties of proposed redox markers and their conjugates with adenosine were investigated in DMF solution of 0.1 M TEAC using gold disc electrode. Before any voltammetric experiments, the gold electrode was polished successively with alumina powder of grain sizes from 1 μm to 0.05 μm. Then the electrode was washed with H_2O and sonicated for 15 min. Next, the surface of working gold disc electrode was treated with piranha solution for 30 s. The solution was removed and the electrode was washed with H_2O. The last step of electrode preparation was voltammetric cycling in buffer solution dedicated to electrochemical measurements, until the characteristic cyclic voltammogram (CV) for a clean gold was obtained.

The DNA recognition layer was prepared as described in Ref. [30]. Briefly, after the electrode cleaning, the 4 μM solution of thiolated ssDNA in IB was dropped on the gold working disc electrode. The ssDNA immobilization has been conducted for 120 min. Then the solution was removed and the electrode was washed in IB. After that, the 1-μM solution of MCH in IB was dropped and left on the electrode surface for 30 min. The electrode was subsequently washed with IB and prepared for hybridization. The hybridization has been carried out for 150 min at ambient temperature. The electrode was then washed with HB and electrochemical experiments were conducted. All measurements were repeated at least three times.

All QCM-D experiments dealing with immobilization and hybridization of DNA were conducted with the same buffer solutions as in the case of electrochemical assays. Nevertheless, to clean the gold transducers used in gravimetric experiments, the QCM sensors were subjected to base piranha solution treatment at 70 °C for 15 min. Next, the sensor was washed with abundant amount of water, absolute ethanol and dried under an argon atmosphere. The sensor was placed in flow cell of QCM-D instruments and the immobilization and hybridization experiments were conducted. The flow was set at 0.1 $mL \cdot min^{-1}$.

3. Results and discussion

There are some important issues that should be considered when choosing the best electrochemical marker for DNA detection. Most DNA biosensors are constructed using self-assembling technique, usually by immobilizing thiol-modified receptor DNA strands on gold electrode. Such receptor layer, although relatively stable, can be damaged if to high electrochemical potential is applied [31]. Thus, relatively low redox potential of the marker is a great advantage. Since appropriately chosen label may allow for good detection limit in biosensor applications, the current signal intensity is also of great importance.

In this work, the usefulness of some redox active metallacarboranes as novel markers for the construction of electrochemical DNA biosensor is evaluated. The carborane complexes containing Fe^{3+}, Co^{3+} or Cr^{3+} cations were examined. The cyclic and square wave voltammetry analysis of 5 mM solutions of each metallacarborane were conducted in DMF with the addition of 0.1 M TEAC as electrochemical medium. Fig. 2 shows SWV only for Fe^{3+} and Co^{3+} metallacarborane, as no current response was detected for Cr^{3+} complex. Both Fe^{3+} and Co^{3+} metallacarboranes show reversible electrochemical properties in CV experiments (data not shown). This promotes its use as redox markers in DNA biosensors design. For the metallacarboranes containing iron and cobalt cations the redox potentials were −0.004 V and −0.988 V, respectively (SWV measurements). As shown in Fig. 2, the current signal of Co^{3+}- (1.636×10^{-5} A) is slightly higher, as compared to Fe^{3+}- (1.585×10^{-5} A) complex.

To further evaluate the usefulness of metal-modified carboranes for DNA biosensors, the conjugates with 2′-deoxyadenosine were prepared and again the electrochemical analysis was conducted (data not shown). Measurements were conducted for 0.01 M solutions in DMF, containing 0.1 M TEAC. The difference in peak height between Fe- and Co-containing conjugates is more significant, as compared to metallacarboranes without adenosine, 4.108×10^{-5} A and 4.673×10^{-6} A respectively. The redox potentials for these compounds were very similar to free metallacarboranes: 0.016 V for Fe^{3+}-complex and −0.965 for Co^{3+}-complex.

Relatively negative redox potential of Co-carborane makes it rather unsuitable in biosensors applications, where self-organized monolayer is required. Accordingly, in subsequent experiments, only Fe-carborane was used.

To confirm the ssDNA monolayer formation and its hybridization with complementary strand, the QCM experiments were conducted. The change in resonance frequency of piezoelectric crystal is the indication of a mass deposited on the transducer. As shown in Fig. 3 (a) after deposition of self-organized monolayer on gold surface and conditions adjustment, hybridization occurs only with fully complementary oligonucleotide (b), as indicated by the frequency change after the addition of **A1** probe. No frequency change was observed when no complementary oligonucleotide **A3** was introduced into QCM system.

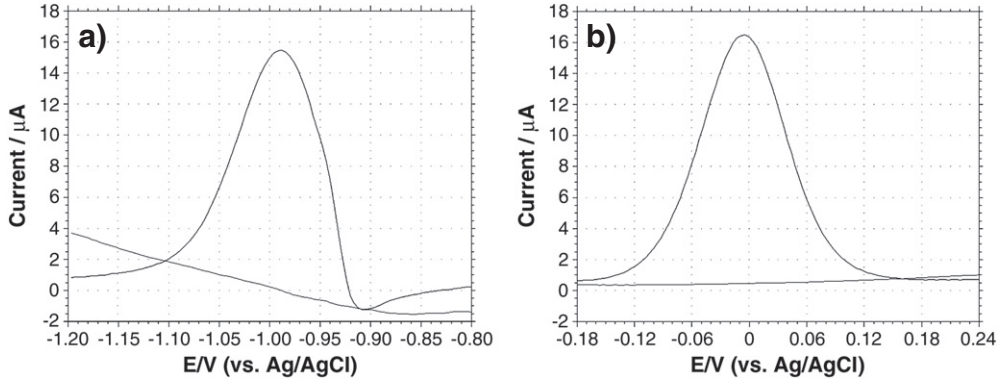

Fig. 2. SWV of 5 mM solutions of a) Co-carborane and b) Fe-carborane in DMF with the addition of 0.1 M TEAC.

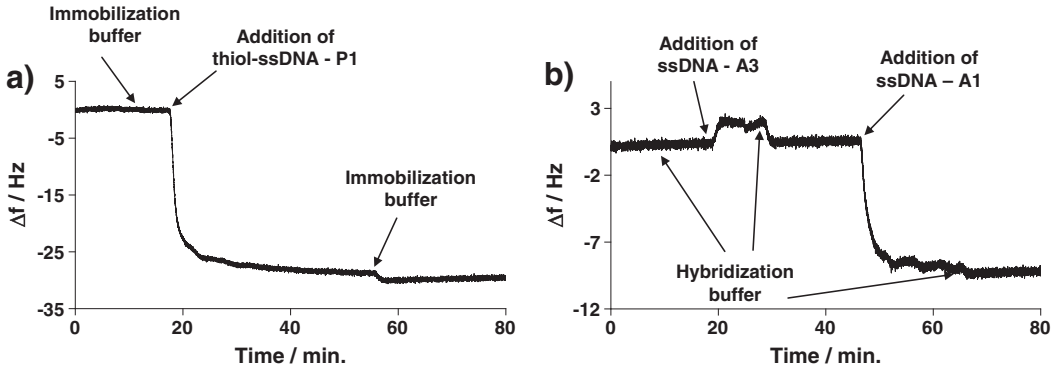

Fig. 3. QCM results of a) immobilization and b) hybridization. All oligonucleotides were dissolved in suitable buffers solutions. $\Delta f = f - f(\mathrm{ref})$, where f(ref) is 14891007 Hz or 14891178 Hz, respectively.

After confirmation of monolayer formation and its hybridization with complementary strand, the electrochemical biosensing measurements were conducted (Fig. 4). The deposition of self-assembled monolayer was performed according to procedure mentioned in Experimental section and described previously [30]. For first experiments, DNA biosensors modified with **P1** DNA probe (see Experimental section) was prepared, while **A1** strand was used as the target (Fig. 4a). The same oligonucleotides were used earlier in QCM measurements. It was found that, after immobilization and hybridization, no current signal was detected with SWV. It should be mentioned that in this configuration, an electrochemical marker (metallacarborane modified with iron) is located close to the electrode surface. To verify these results, DNA biosensor was prepared with **P2** oligonucleotide, where the –SH moiety was attached at 5′ end, instead of 3′ as was in case of **P1** probe (Fig. 4b). In this arrangement, after the hybridization with **A1**, marker is located at the end of resulting dsDNA fragment, relatively far from the electrode surface. Somewhat surprisingly, this configuration allowed for detection of a current signal (Fig. 4b). To make sure that observed current is indeed related with Fe-carborane presence in the analyte DNA sequence, SWV measurements for sensors modified with **P1** or **P2** probes and **A2** target oligonucleotide (the same sequence as **A1**, containing no redox marker) were conducted. No signal was observed for both types of sensor (for SWV voltammogram recorded on sensor with **P1** probe, see Fig. 4c), which confirms that Fe-carborane acts as an electrochemical marker of DNA hybridization.

Results presented above, obtained for **A1** oligonucleotide and **P1** (−SH moiety at 3′ end) or **P2** (−SH moiety at 5′ end) capture probes, are interesting and require further explanation. The fact that **A1**/**P2** receptor/target pair, with Fe-carborane located away from the electrode, induces voltammetric response after hybridization, is not itself surprising. Possible explanation of this phenomenon, reported in recent literature, is the ability of dsDNA to act as a conductor, allowing electron transfer from the electrode surface to the redox marker [32]. The lack of current signal for **A1**/**P1** receptor/target pair, where Fe-carborane is situated near the electrode surface, was rather unexpected for us. However, similar behavior was observed by Di Giusto et al. [33]. It was reported that the electrochemical response of ferrocene-labeled nucleoside triphosphate was the most pronounced when the marker was attached at the distal end of dsDNA. The current signal was approximately five times lower for the same marker attached at the proximal end, close to the electrode surface. No explanation related to the origin of such a behavior was provided, thus this phenomenon should be further explored.

As it can be seen in Fig. 4b), the current signal of Fe-carborane consists of two discrete peaks, at −0.41 V and −0.49 V. Similar results were obtained in the case of determination of traces of heavy metal cations by anodic stripping voltammetry at noble metal electrodes. This was attributed to the deposition of the analyte into

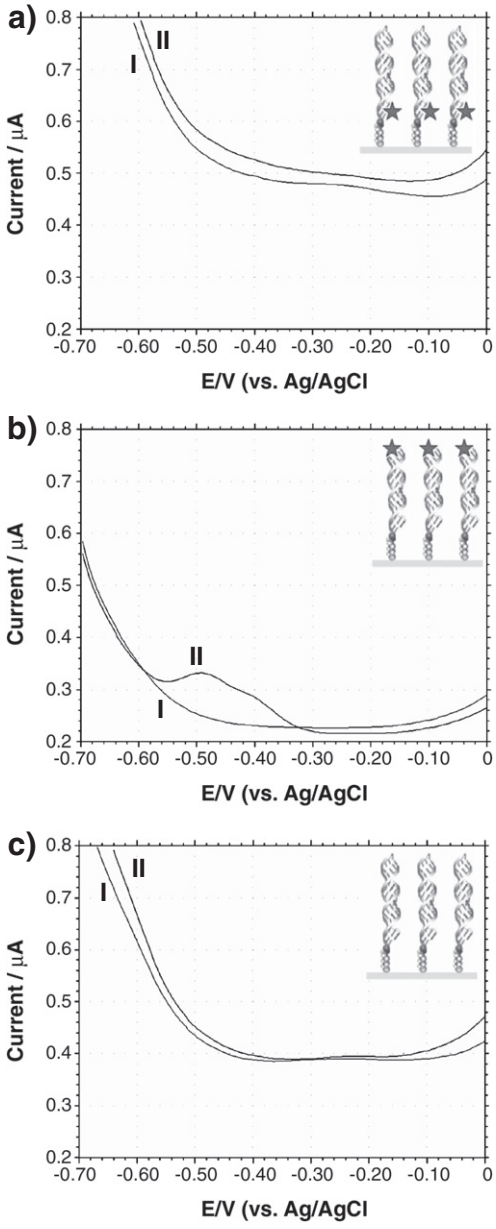

Fig. 4. SWV for oligonucleotide-modified sensors: a) P1 probe with A1 target; b) P2 probe with A1 target; c) P2 probe with A2 target. I) SWV after immobilization; II) SWV after hybridization. Inserts show schematic configuration of each system; ★- metallacarborane label.

multiple states of activity with differing degrees of interaction with the electrode [34]. It can be postulated that Fe-carborane modified oligonucleotide can form a monolayer of certain heterogeneity, with regions of different orderliness. Accordingly, the strength of interaction of the redox label with electrode surface varies, leading to the appearance of multiple peaks.

4. Conclusions

Complexes of carboranes with Co^{3+}, Cr^{3+} and Fe^{3+} metal cations were evaluated as electrochemical markers for the development of DNA hybridization biosensors. Based on cyclic and square wave voltammetry investigations of tested metallacarboranes, as well as of their conjugates with 2′-deoxyadenosine, it was shown that only Fe-carborane fulfill the requirements for redox label. DNA sensors were prepared using gold electrodes as transducers, with receptor layer formed by self-assembling of thiol-modified oligonucleotides. The aqueous solution of target DNA strand, modified with Fe-carborane, was placed onto the electrode to allow hybridization. This process, as well as earlier immobilization of capture probes, was monitored using quartz crystal microbalance and square wave voltammetry measurements. It was found that molecular recognition, i.e. hybridization between receptor layer and target DNA, takes place if these two strands are complementary. However, electrochemical signal can be observed only for a particular sensor configuration, with the marker located away from the electrode after the hybridization. For reversed sensor arrangement, where the marker was situated close to the electrode surface after the molecular recognition, no current signal was observed. Efforts are currently in progress in our laboratories to elucidate this apparently surprising behavior. However, based on the results of our investigations, it can be concluded that Fe-carboranes can be applied as useful markers for electrochemical DNA sensing.

Acknowledgments

This work was supported in part by grants: K152/H03/2007/10, N204 531739 and N204 125237 from the Polish Ministry of Science and Higher Education and by an internal grant from the Institute for Medical Biology PAS.

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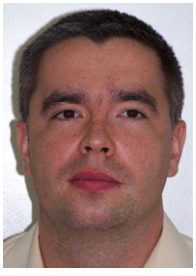

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