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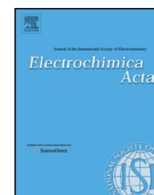

Synthesis and electroactivated addressing of ferrocenyl and azido-modified stem-loop oligonucleotides on an integrated electrochemical device

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ABSTRACT

We report a strategy to address stem-loop oligonucleotides on a gold surface in order to develop a robust and ultra-sensitive integrated electrochemical DNA sensor. Probe immobilization relies on the potential-assisted copper-catalyzed alkyne-azide cycloaddition. Firstly, a tetrathiol-hexynyl derivative was used to introduce alkyne functions onto the electrode surface. This anchor has proved its robustness in conditions used for the “click” reaction and in wet storage. Then, different ferrocenyl and azido-modified stem-loop oligonucleotides were synthesized using the solid-phase synthesis technique and their immobilization was studied. Hybridization assays with the DNA target were performed in a complex medium by cyclic voltammetry. The detection sensitivity achieved by our functionalized electrodes was significantly increased, as a detection limit of 10 fM was determined. We also demonstrated that grafting of the stem-loop oligonucleotides *via* the electroactivated “click” reaction was specific to the gold surface on a microfabricated electro chemical device for the Lab-on-Chip application that fully integrates Au working microelectrodes, Pt counter and Ag reference electrodes.

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1. Introduction

Nucleic acids are precious biological tools that are frequently used to elaborate analytical devices for medical diagnostics. Their sequence-specific binding properties are exploited to control either their architecture, for instance by way of a stem-loop structure, or patterning on a support by self-assembly, as for DNA origami [1]. The conformational change of a DNA probe induced by the binding process is frequently exploited in the development of electrochemical DNA sensors. Fan et al. [2] and Immoos et al. [3] pioneered the use of ferrocene (Fc) modified stem-loop DNA as capture probes for sensors. Probe binding with its complementary DNA target induces a conformational change which is monitored using electrochemical methods. Liu et al. also developed a ferrocene-modified DNA sensor for the electrochemical detection of hepatitis C virus based on site-specific DNA cleavage of an endonuclease [4].

In the literature, many strategies have been described to synthesize Fc-modified DNA conjugates [5]. Hüsken et al. reported the synthesis of “four-potential” ferrocene labeling of PNA oligomers *via* click reaction [6]. In our laboratory, we have developed strategies for modifying oligonucleotide sequences with many ferrocene derivatives directly during DNA automated solid-phase synthesis [7–9]. Stem-loop DNA labeled with four Fc molecules was synthesized and used as a probe in a gold electrode microsensor to accomplish DNA target detection [10]. A detection limit of 3.5 pM was achieved by this DNA sensor.

Electrochemical methods can be easily adapted to a multiplexed format. The addressing of probes on a multi-detection system is a crucial step that directly impacts biochip performance.

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Devaraj et al. first described the addressing of a ferrocene derivative on independent gold microelectrodes by electrochemical activation of the alkyne/azide cycloaddition (Sharpless “click” reaction) [11]. This group introduced a method in which the active Cu(I) catalyzing the 1,2,3-triazole formation between a terminal alkyne and an organic azide was selectively and locally generated under a negative potential on the electrode. They demonstrated that this technique can provide a spatial resolution of the grafting reaction [12]. Canete and Lai reported the elaboration of a multi-detection system by addressing stem-loop oligonucleotides (ON) on an electrode array using the Cu(I) electrocatalyzed “click” reaction [13]. A three-probe DNA sensor was developed that could simultaneously detect three different DNA targets at a concentration of 1 μM. Furst et al. recently reported the same grafting method for DNA patterning on a two-electrode platform [14].

In our laboratory, we have developed a new approach to produce robust sensor arrays *via* Cu(I) electrocatalyzed azide/alkyne cycloaddition [15]. It consists of grafting a tetrathiol anchor onto gold electrodes to enhance the stability of the tethered entities in the buffer and to allow efficient surface coverage. A bis (dithiolphosphate) hexynyl derivative ($(DTPA)_2$hexynyl linker) was synthesized. The compound provided a tetra-thiol bound on a gold electrode. The alkyne function of the linker enabled the “click” reaction. The improved stability provided by this multidental anchor was confirmed, allowing several regenerations of the sensor.

In this paper, we focus on addressing ferrocene-modified stem-loop oligonucleotides on gold electrodes *via* the Cu(I) electrocatalyzed azide/alkyne “click” reaction. An electrochemical characterization of the functionalized electrodes is presented. A hybridization study enabled us to determine a detection limit for the complementary DNA target at 10 fM, which is much better than the sensitivity achieved by previously described stem-loop DNA sensors developed using different grafting strategies [10,16].

We also confirmed the robustness of the sensitive layer on the sensor which allowed several regenerations of the system. Finally, we investigated the electrocatalyzed “click” reaction on an integrated electrochemical device, comprising a gold working microelectrode of 19.6×10^{-4} mm^2, a platinum counter electrode of 1 mm^2 and an Ag/AgCl reference electrode of 2×10^{-2} mm^2. A fluorescent azide-modified oligonucleotide was electro addressed following our protocol. Fluorescence recorded by microscopy confirmed the selective functionalization of the gold surface.

2. Experimental

2.1. Chemicals

Potassium phosphate monobasic ($\geq$98%), sodium perchlorate and tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]amine (TBTA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP/HCl), all other solvents and chemicals were purchased from Sigma-Aldrich. Sodium phosphate dibasic dihydrate ($\geq$99%), celite and silica gel were obtained from Fluka. Sodium hydroxide was obtained from Laurylab. Phosphoramidites, dT-CPG column, reagents and solvents used for DNA synthesis were purchased from Glen Research (Sterling, Virginia). The salmon sperm DNA (10 mg/mL) was purchased from Invitrogen. The non-complementary target (5’ TTT TTA TTG AGA TTC CCG AGA TTG ATT TTT 3’) and the complementary influenza target (5’ TTT TTTTTT TAG TTT TTG GAC GTC TTC TCC TTT TTTTTT T 3’) were obtained from Eurogentec. The fluorescent (Cyanine5) oligonucleotide (5’ $C_6H_{12}N_3$-TTT TTT TTT T Cy5 G 3’) was synthesized in the laboratory using standard DNA solid-phase synthesis methods. ESI Mass Spectrometry was conducted on a Bruker micrOTOF-Q II and MALDI-TOF MS on an Applied Biosystems Voyager DE-PRO (Foster city, USA), using 2,4,6-trihydroxyacetophenone monohydrate (THAP) as the matrix. ^{1}H NMR spectra were recorded on a DRX 300 Bruker spectrometer.

2.2. Synthesis of the $(DTPA)_2$hexynyl linker

The $(DTPA)_2$hexynyl linker was synthesized by a solid-phase approach according to a protocol previously described [15]. The purity was controlled by high performance liquid chromatography and a global yield of 80% was estimated by UV spectroscopy.

2.3. Synthesis of the oligonucleotides $(DTPA)_2$In4Fc, N_3In’1Fc and Tb $(N_3)_3$In’4Fc

The oligonucleotides were synthesized with an automated instrument *via* the phosphoramidite chemistry, according to a standard protocol. A dT-CPG column was used to start each synthesis. All the resulting oligonucleotidic sequences ended with a thymidine at the 3’ extremity. The synthesis was achieved *via* the standard 1 μM phosphoramidite cycle on the synthesizer. The bifunctional ferrocene phosphoramidite (1-[3-*O*-dimethoxytrityl-propyl]-1-[3-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidityl) propyl]ferrocene) was used in the DNA synthesizer to incorporate Fc directly into the oligonucleotide sequence during solid-phase synthesis. A coupling time of 30 seconds was applied for the incorporation of the ferrocene synthon (0.1 M in acetonitrile (AcCN)). For the $(DTPA)_2$In4Fc, two successive incorporations of the dithiolphosphoramidite synthon (DTPA) were achieved at the end of synthesis, following the protocol recommended by Glen Research. For the N_3In’1Fc, the 5’-bromohexyl phosphoramidite was incorporated at the 5’ extremity with a coupling yield >98%. For the Tb$(N_3)_3$In’4Fc, the trebler synthon (Tris-2,2,2-[3-(4,4’-dimethoxytrityloxy) propyloxymethyl]ethyl-[(2-cyanoethyl)-(N, N-diisopropyl)]phosphoramidite) was incorporated at the 5’ extremity with a coupling yield of 85% before coupling the bromohexyl phosphoramidite. The N_3In’1Fc and Tb$(N_3)_3$In’4Fc functionalized CPG were then suspended in 2 mL of a solution of sodium azide (100 mM), sodium iodide (100 mM) in anhydrous DMF in order to substitute the bromo functions to azides. The reaction was performed for 2 hours at 65 °C. After the cleavage and deprotection step in 1 mL of concentrated NH_4OH (30%/H_2O), 16 hours at 60 °C, the crude materials were concentrated to dryness in a speed-vacuum instrument. The oligonucleotides were purified by HPLC using a DeltaPak C18 15 μm 300 Å (300 $\times$ 7.8 mm) column with an acetonitrile gradient from 0 to 50% in 0.05 M triethylammonium acetate buffer (TEAAc), pH 7. Oligonucleotide purity was controlled by HPLC using an X-terra MS C18 2.5 μm (50 $\times$ 4.6) column from Waters (Versailles, France). Analyses were carried out with an acetonitrile gradient from 5 to 50% in 0.05 M TEAAc, pH 7, in 65 min at 60 °C. Then, the oligonucleotide was characterized by MALDI-TOF MS analysis $(M-H)^-$ (g mol^{-1}): 10185.2 for $(DTPA)_2$In4Fc (Calculated: 10187.49); 10771.0 for N_3In’1Fc (Calculated: 10773.8); 12641.9 for Tb$(N_3)_3$In’4Fc (Calculated: 12646.4).

2.4. Electrochemical materials

Two different silicon-based technologies were developed for the fabrication of gold electrodes using LAAS-CNRS clean room facilities. First, planar gold square electrodes were produced. Starting with an oxidized silicon substrate (silicon oxide thickness: $\sim$ 0.3 μm), a 300 nm gold layer was evaporated on a 10 nm titanium under layer to elaborate a Si/SiO_2/Ti/Au structure. Specific attention was given to the deposit of low-stress films. Thus, gold adhesion on silicon oxide SiO_2 was ensured without thermal annealing, preventing titanium atoms from diffusing towards the upper electro-active gold surface. Finally, the Si/SiO_2/Ti/Au

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