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Selective functionalization of Au electrodes by electrochemical activation of the "click" reaction catalyst

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ABSTRACT

The potential-assisted copper-catalyzed alkyne–azide cycloaddition was investigated to modify a gold electrode surface. Firstly, a tetrathiol-hexynyl derivative was used to introduce alkyne functions on the surface. This anchor proved its robustness in conditions used for the "click" reaction and in wet storage. Then, the potential-assisted "click" reaction was studied with an azido ferrocene derivative. The experimental conditions were optimized according to the electrochemical response of the sensor by cyclic voltammetry and the "click" reaction yield was established. After functionalization, the presence of the 1,2,3-triazole group was confirmed by XPS. A fluorescent azido oligonucleotide was grafted onto the gold surface allowing visualization of the reaction by fluorescence microscopy.

1. Introduction

With the aim of developing rapid and selective analytical systems, much research is currently devoted to the elaboration of microarrays which allow real-time, label-free and multiplexed detection [1]. In the context of manufacturing a portable device, electrochemical sensors are considered the cutting edge of this domain [2]. The literature describes many of these systems [3] which are based either on electrochemical impedance spectroscopy measurement [4–7] or on voltammetry measurement thanks to the presence of a redox molecule in the sensitive layer of the electrodes [8–12]. Ferrocene (Fc) is widely used as a redox label and has already proven its efficiency as such [13,14]. A wide variety of Fc-oligonucleotide conjugates have been described for the electrochemical detection of DNA [15–23]. Fan et al. [23] and Immoos et al. [15] pioneered the use of Fc modified stem-loop DNA as a capture probe for sensors. In our laboratory, we have developed a strategy for modifying oligonucleotide sequences, with many ferrocene derivatives, directly during automated DNA synthesis [18]. A 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 [24–26]. 1 pM sensitivity was achieved for the detection of a long chain analog of DNA PCR products [26]. The system was also able to differentiate single-mismatches in the DNA target.

Electrochemistry is also a method that can be easily adapted to a multiplexed format. The addressing of probes on a multidetection system is a crucial step that directly impacts biochip performance. For this purpose, much research has been devoted over the past decades to the electro-addressing of probes on a multi-electrode array. Electropolymerization of pyrrole, for instance, has proved to be an efficient method for addressing DNA on miniaturized electrodes [27–29]. A Ppy film can be easily generated on electrodes by well-controlled polymerization. Other groups have focused on developing strategies that avoid the presence of a polymer film between the DNA probes and the support. Aryl diazonium salt chemistry appears promising for achieving covalent grafting on carbon and gold surfaces by electroactivation. Corgier et al. described the grafting of aryl diazonium modified biomolecules, such as DNA or proteins, onto both polarized carbon [30,31] and gold [32] electrodes. The reduction of the aryl diazonium moiety achieved under a negative potential formed a covalent C—C or C—Au bond between the aryl group and the electrode. More recently, Devaraj et al. described the addressing of independent gold microelectrodes by electrochemical activation of the alkyne/azide cycloaddition (sharpless "click" reaction) [33]. This group introduced a method by which the active copper(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

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of the grafting reaction [34]. Canete and Lai reported the elaboration of a multi-detection system by addressing hairpin probes onto an electrode array through the copper(I) electrocatalyzed alkyne/azide "click" reaction [35]. However, this strategy relies on thiol chemistry for grafting onto the gold surface and it is well known that the thiol monolayer is moderately stable on a gold surface since the binding energy of the Au—S bond is only about 30–45 kcal mol^{-1} compared to at least 100–150 kcal mol^{-1} for a covalent C—C bond [36]. The Au—S bond tends to oxidize in air and media, to decompose upon temperature in an aqueous solution and has a low potential stability window, which limit the usefulness of this chemistry for biosensors.

In light of the works mentioned above, we investigated an alternative way of immobilizing probes on gold surfaces. We focused on the DTPA (dithiolphosphoramidite) moiety, which enables the stability of the grafted probes to be enhanced over time and achieves efficient surface coverage. The incorporation of two (4 thiols) or three (6 thiols) DTPA at the probe's extremity increased the anchoring stability of the probes during the passivation step with mercaptohexanol and dehybridization treatment using NaOH [36].

Considering this result, we synthesized a $(DTPA)_2$-alkyne derivative in order to obtain a strong anchoring of this compound on the gold surface of the sensor to achieve the "click" reaction by electrochemical activation. An original strategy of solid-phase synthesis of the molecule was implemented. Then, the electrochemical activation of the 1,3-cycloaddition between alkyne and azide functions was studied. The "click" reaction was first carried out with an azido ferrocene derivative. The electrochemical response of the sensor was monitored by cyclic voltammetry and experimental conditions were determined. Then, the same reaction was achieved with a fluorescent azido oligonucleotide and the grafting efficiency was confirmed by fluorescence microscopy. The method developed in this work can potentially provide robust and selective immobilization of DNA probes on a gold surface for the design of a multi-addressed microarray.

2. Experimental

2.1. Chemicals

The phosphoramidites, reagents and solvents used for DNA synthesis were purchased from Glen Research (Sterling, Virginia). Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and all other solvents and chemicals were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Celite and silica gel were purchased from Fluka (France). Thin layer chromatography (TLC) was carried out on aluminum sheets coated with silica-gel 60 F (Merck 5554). TLC plates were revealed by UV light and charring after spraying with 5% H_2SO_4 in EtOH. ESI Mass Spectroscopy was conducted on a Bruker micrOTOF-Q II and MALDI-TOF MS on an Applied Biosystems Voyager DE-PRO. ^{1}H NMR spectra were recorded on a DRX 300 Bruker spectrometer. The chemical shifts were expressed on the δ scale in parts per million (ppm). The following abbreviations were used to explain the observed multiplicities: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quadruplet; m, multiplet; br, broad.

2.2. Synthesis of 2-azidoethyl ferrocene **7**

2.2.1. Synthesis of N,N,N-trimethyl-1-ferrocenylmethanaminium iodide **1**

The methyl iodide (512.2 mmol, 3.5 mL) was added dropwise to a mixture of N,N-dimethylaminomethylferrocene (10.43 mmol, 2.5316 g) in methanol (50 mL) under an argon atmosphere. After stirring for 3 h at room temperature, the solution was dried *in vacuo*. Afterwards, the residue was dissolved in a minimum amount of methanol and 400 mL of diethyl ether was introduced to precipitate the compound. After filtration, the product was rinsed with diethyl ether. Compound **1** was obtained with a yield of 96% as a yellow powder (10.00 mmol, 3.8502 g).

^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.32 (s, 9H, H_6), 4.31 (s, 5H, H_4), 4.34 (d, 2H, H_2), 4.59 (m, 2H, H_3), 4.92 (s, 2H, H_5 and $H_{5'}$). MS (ESI+) *m*/*z*: calcd for $C_{14}H_{20}FeN^+$ $[M+H]^+$ 258.1, found 257.9.

2.2.2. Synthesis of 2-ferrocenylacetonitrile **2**

Compound **1** (6.18 mmol, 2.3793 g) was added to a solution of potassium cyanide (66.43 mmol, 3.3215 mg) in 30 mL of water, in a two-necked flask equipped with a reflux condenser and the mixture was heated overnight with steady stirring. The solution was cooled at room temperature for a couple of minutes, and then filtered. The residue was extracted three times by 50 mL of diethyl ether. The combined ethereal solutions were washed with water. Evaporation of solvents *in vacuo* yields compound **2** as a light brown solid (4.55 mmol, 1.0238 g, 74%).

^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.42 (s, 2H, H_5 and $H_{5'}$), 4.18 (d, 2H, H_2), 4.24 (s, 5H, H_4), 4.26 (d, 2H, H_3). MS (ESI+) *m*/*z*: calcd for $C_{14}H_{20}FeN^+$ $[M+H]^+$ 225.0, found 225.9.

2.2.3. Synthesis of 2-ferrocenylacetic acid **3**

Compound **2** (4.12 mmol, 926.6 mg) was dissolved in 9 mL of ethanol and added to a solution of potassium hydroxide in water (2 M, 23 mL). The mixture was kept under stirring and at reflux for 12 h. The solution was dried *in vacuo*, diluted in 25 mL of water and washed twice with 10 mL of diethyl ether. Then, hydrochloric acid (2 M, 23 mL) was introduced dropwise under stirring in the aqueous layer to precipitate the compound. The precipitate was filtered and washed three times with 12 mL of cool water, to obtain the expected product as a yellow powder (3.34 mmol, 812.0, 81%).

^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.40 (s, 2H, H_5 and $H_{5'}$), 4.08 (s, 7H, H_3 and H_4), 4.05 (s, 2H, H_2). MS (ESI+) *m*/*z*: calcd for $C_{12}H_{12}FeO_2$ $[M+H]^+$ 244.0, found 244.0.

2.2.4. Synthesis of 2-ferrocenyl ethanol **4**

A solution of lithium aluminum hydride (6.5 mmol, 247.0 mg) in 20 mL of anhydrous tetrahydrofurane was cooled under stirring at 0 °C in an ice bath. Afterwards, compound **3** (3.25 mmol, 792.2 mg) was dissolved in 15 mL of anhydrous THF and added dropwise to the cooled solution. The system was left at room temperature for a couple of minutes, and then kept at reflux for 1 day. After the reaction, the solution was cooled in an ice bath and 20 mL of water was introduced slowly. After filtration on celite, the solution was extracted 3 times with 35 mL of diethyl ether. Organic layers were reassembled and dried *in vacuo* to obtain compound **4** as an orange crystalline solid (2.42 mmol, 606.0 mg, 75%).

^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 2.68 (s, 2H, H_5 and $H_{5'}$), 4.05 (s, 9H, H_2, H_3 and H_4), 4.12 (s, 2H, H_6 and $H_{6'}$). MS (ESI+) *m*/*z*: calcd for $C_{12}H_{14}FeO$ $[M+H]^+$ 230.0, found 229.0.

2.2.5. Synthesis of 2-ferrocenylethan 4-methylbenzenesulfonate **5**

Triethylamine (0.60 mL, 4.30 mmol) was added to 3.5 mL of a solution of **4** (549.8 mg, 2.5 mmol) in CH_2Cl_2. The reaction mixture was cooled to 0 °C and p-toluenesulfonyl chloride (988.1 mg, 5.19 mmol) was added. The reaction mixture was stirred for 3 h at 40 °C, then diluted in 15 mL of Et_2O and successively washed with 10 mL of water, 5 mL of a 0.4 M HCl solution, 5 mL of saturated aqueous $NaHCO_3$ and finally 5 mL of saturated aqueous NaCl. The organic layer was dried over sodium sulfate and concentrated *in vacuo*. The residue was purified by flash chromatography on a silica gel column (cyclohexane/triethylamine 99:1 then cyclohexane/MeOH 90:10)