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Label-free electrochemical monitoring of protein addressing through electroactivated "click" chemistry on gold electrodes

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ABSTRACT

In this work, using electrochemical impedance spectroscopy (EIS), we have, for the first time, label-free monitored protein immobilization on a gold surface through a strategy of electroaddressing, compatible with the production of microarrays for multi-detection. This functionalization is achieved via the alkyne/azide cycloaddition, better known as the "click" reaction. The electroaddressing was applied to a polythiol hexynyl derivative previously grafted onto the gold surface. This compound consists of two dithiol phosphate groups and a hexynyl function and was synthesized through a supported synthesis approach, from a dithiol reagent, phosphoramidite (DTPA), and a hexynyl phosphoramidite. Next, an azide-PEG3-biotin derivative was grafted onto the modified gold surface by electro-chronocoulometry. The "click" reaction was controlled by electrochemical impedance spectroscopy, showing the change in impedance only when the electroaddressing was performed at −300 mV. No effect on the EIS signal was observed when a positive potential was applied, confirming the specificity of the electroactivation. Biotin-modified electrodes were used to fix streptavidin and the immobilization was monitored using EIS. Fluorescent streptavidin-functionalized silica nanoparticles were also specifically grafted onto the biotinylated gold surface in order to confirm the "click" reaction using fluorescence microscopy. The obtained streptavidin platform was used to detect the surface coverage by biotinylated human serum albumin (HSA). The lowest detectable concentration is 10 pg/mL, and surface saturation is obtained with concentrations higher than 100 ng/mL.

1. Introduction

In affinity biosensors, an immobilized recognition bioelement (probe) selectively binds to the analyte (target) molecule. The detection of the binding event (target/probe) is based on the change of an electrical parameter at the electrode/electrolyte interface when an electrochemical transducer is used. These transducers can be potentiometric [1], amperometric [2], conductometric [3] or impedimetric [4], converting the biochemical information into a measurable signal. Electrochemical biosensors have recently received particular attention since they possess a number of attractive characteristics: they are label free, low cost, require cost-effective instrumentation, can be mass produced, miniaturized and integrated into multi-array or microprocessor-controlled diagnostic tools, etc. Focusing on impedimetric biosensors, they allow the detection of biological targets without any previous labeling step (fluorophores, redox enzyme, etc.) of the biomolecules [5]. Furthermore, impedimetric techniques have been used to characterize the fabrication of biosensors and to monitor the catalyzed reactions of enzymes or the biomolecular recognition events of specific binding proteins, lectins, receptors, nucleic acids, whole cells, antibodies or antibody-related substances [6].

The crucial stage in biosensor manufacture/design remains the choice of the method of immobilization of the biological material on the electrode. The most commonly used biomolecule immobilization techniques for designing specific biosensors are physical adsorption, entrapment, intermolecular cross-linking and covalent binding [7]. Alongside these widespread immobilization techniques, electrochemical methods of immobilization and in particular electro-addressing methods arouse particular interest as new approaches to fix active biomolecules on the surface of a conductive material. Applying an electrochemical potential to the specific electrode (chip) allows biomolecule immobilization, which should be compatible with the production of biochip microarrays for multi-detection.

For this purpose, much research has been devoted over the past decades to the electro-addressing of probes on a multi-electrode array. The electropolymerization of pyrrole, for instance, has proved to be an efficient method for addressing DNA on miniaturized electrodes [8–11]. Aryl diazonium salt chemistry appears promising for achieving covalent grafting on carbon and gold surfaces by electroactivation.

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Corgier et al. [12,13] described the grafting of aryl diazonium-modified biomolecules, such as DNA or proteins, onto both polarized carbon and gold [14] 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.

With the appearance of the "click" chemistry first proposed by Sharpless in 2001 [15], the copper(I)-catalyzed azide-alkyne cycloaddition reaction becomes a particularly powerful tool to modify biomolecules or to tether them on inorganic materials. It has also been used for the fluorescent detection of histidine through the inhibition of copper(II)-induced ascorbate oxidation by histidine [16]. The "click" reaction displays several important criteria, such as high efficiency under mild conditions, minimal by-products and limited side reactions [17,18].

More recently, Devaraj et al. [19] described the addressing of independent gold microelectrodes by electrochemical activation of the alkyne/azide cycloaddition (Sharpless "click" reaction). 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 of the grafting reaction [20]. Bard and co-workers [21] used this technique for surface patterning with fluorescent molecules by SECM. Canete and Lai [22] 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. However, this strategy relies on thiol chemistry for grafting onto the gold surface, and it is well known that a 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 compared to at least 100–150 kcal/mol for a covalent C-C bond [23]. The Au-S bond tends to oxidize in air and media, to decompose under a temperature increase in an aqueous solution, and has a low potential stability window, which limit the usefulness of this chemistry for biosensors. Then we focused on the dithiol phosphoramidite (DTPA) moiety, which enables the stability of the grafted probes to be enhanced over time and achieves efficient surface coverage. The incorporation of two (4 thiol) DTPA increased the anchoring stability for further biofunctionalization steps [24]. Recently, Qi et al. [25] has reported a double electrochemical coupling method for addressing antibodies on a carbon ink electrodes.

The efficiency of the potential-assisted copper-catalyzed "click" reaction was previously determined using or fluorescent molecules [21,26,27] or redox probes, such as ferrocene [28,29]. In this work, the potential-assisted copper-catalyzed "click" reaction is used for the specific immobilization of a protein molecule on a gold chip and each step of the procedure was monitored, for the first time, using a label-free technique, EIS.

2. Materials and methods

2.1. Chemicals

Azide-PEG3-biotin conjugate was bought from Jena Bioscience (Saint-Martin-d'Hères, France). Tris(((1-benzyl-1H-1,2,3-triazol-4-yl) methyl))amine (TBTA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, HCl), streptavidin and all solvents and chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Streptavidin-functionalized rhodamine-doped silica nanoparticles [30] were provided by Nano-H (Saint Quentin Fallavier, France). Biotinylated human serum albumin protein (biotinylated HSA) was purchased from Abcam (Paris, France). Dithiol phosphoramidite (DTPA) and all phosphoramidites were purchased from Glen Research (Sterling, Virginia, USA).

Bis(DTPA)hexynyl was synthesized using an Applied Biosystems 394 RNA/DNA synthesizer (Applied Biosystems, Foster City, USA) using a protocol described in a previous paper [24].

2.2. Equipment

2.2.1. Electrochemical experiments

Aqueous buffers were made with deionized water purified through a Milli-Q system (Millipore, Bedford, MA). A standard three electrode electrochemical setup was used in a Faraday cage. The counter-electrode was a platinum plate (surface area 0.54 cm^2), and the working electrode was a gold electrode (surface area 0.19 cm^2). When working on an aqueous buffer, especially for the EIS measurements, the reference electrode used was a saturated calomel electrode (SCE), whereas a platinum wire was used in DMSO solutions. Measurements were performed using a Voltalab 80, model PGZ 402, assisted by Voltamaster 4 software. All electrochemical experiments were carried out at room temperature in PBE (phosphate-buffered electrolyte: sodium phosphate (20 mM), $NaClO_4$ (250 mM), pH 6.4). Solutions were deoxygenated under argon before use.

For the electrochemical impedance spectroscopy (EIS), measurements were carried out in the presence of a reversible 10 mM equimolar $Fe(CN)_6^{3-/4-}$ redox probe in phosphate-buffered saline (PBS) (10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4). The measurements were carried out over a frequency range of 100 kHz to 100 mHz at an applied potential of −200 mV using a modulation voltage of 10 mV.

The Nyquist plots were modeled according to the simplified Randles circuit (cf. Scheme 1), using Zplot/Zview Software (Scribner Associate Inc, Southern Pines, NC, USA). The decomposition of the Nyquist diagram into several electrical components (resistance, capacitance) helps to explain the phenomena of mass transfer, electron transfer or diffusion on the surface of modified electrodes. The diameter of the semicircle is related to the charge transfer resistance (R_{ct}) of the layer, which shows its behavior against the electron transfer with the redox couple.

2.2.2. Other characterization techniques

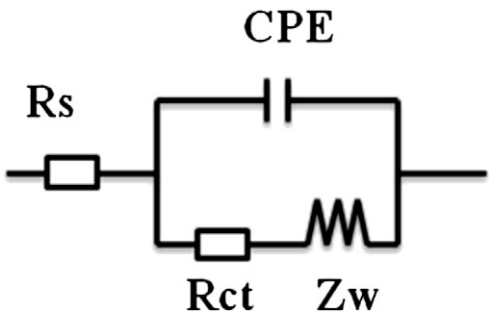
Fluorescence images were taken using a fluorescence microscope (Zeiss Axioplan 2 Imaging apparatus, equipped with × 10 and × 40 lenses and a monochromatic camera).

TEM analyses were performed with a Philips CM120 operating at 80 kV (Microscopy Center of Lyon1 University).

The zeta potential measurement and the hydrodynamic nanoparticle analysis were performed on a Malvern NanoZS Zetasizer instrument using a universal dip cell at 25 °C.

2.3. Synthesis of bis(DTPA) hexynyl derivative

The *bis*(DTPA)-hexynyl derivative is a compound made up of two successive 1,2-dithiane-4-ol-5-phosphate moieties and a hexynyl group bound to the second phosphate group. This molecule was synthesized through a solid-phase strategy using dithiol phosphoramidite (DTPA) and hexynyl phosphoramidite, on a 394 Applied Biosystems DNA synthesizer (Foster City, USA) according to a method described in



Scheme 1. Simplified Randles electrical circuit.