Biochips

DOI: 10.1002/anie.200605010

A Versatile Method for Direct and Covalent Immobilization of DNA and Proteins on Biochips

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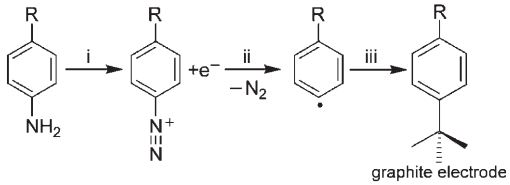
The immobilization of biological molecules is a crucial step in biochip research because it is directly related to the biosensing performances obtained. To date, even though a wide variety of biochips have been developed,[1] no generic procedure has emerged that could be easily applied to both proteins and nucleic acids, and more generally to interaction-based biochips. Nowadays, an obvious need exists to develop a flexible immobilization process to provide strong, stable, and accessible binding of the sensing element, thus leading to sensitive and reproducible biochip performances.

We have recently[2] presented an immobilization strategy that enables the direct grafting of aryl diazonium modified noncatalytic proteins (antigens) onto the surface of screen-printed graphite electrode (SP) biochips (see the Supporting Information). This approach leads to spatially resolved grafting of proteins onto conducting surfaces. The biomolecules were first modified with aniline derivatives, which were oxidized into aryl diazonium functions prior to the electroaddressing.

This technique is based on the particular electrochemical property of aryl diazonium salts.[3] These molecules could be electroaddressed at the surface of a polarized electrode to form a covalent C–C bond between the aryl group and the electrode material (Scheme 1). This electrochemical-grafting property of aryl diazonium derivatives was previously confirmed on a large variety of conductive and semiconductive materials, such as carbon, metal, silicon, diamond, and recently indium tin oxide (ITO) electrodes.[4–8]

Herein, we present a proof of concept of this unique electroaddressing procedure for the immobilization of biomolecules as different as antibodies, nucleic acids, and enzymes. The potential of the electroaddressing chemistry is illustrated with different biosensing architectures (oligonucleotide-based assays, capture immunoassays, and sandwich immunoassays), all involving a chemiluminescent detection system with horseradish peroxidase as label.



Scheme 1. Aniline derivative electroaddressing mechanism: i) diazotation of the aniline derivative, ii) electroreduction of diazonium at a conductive electrode surface, iii) covalent grafting to the carbon electrode surface through a C–C bond.

The first demonstration of an analytical application of the electroaddressing of aryl diazonium modified biomolecules is based on oligonucleotide-functionalized biochips. To our knowledge, only a few works deal with the direct covalent binding of oligonucleotides on conducting material.[7,9] Herein, a 20mer sequence from a "hot spot" of the exon 8 of the p53 tumor suppressor gene[8] was functionalized with 4-aminobenzylamine (4-ABA), electroaddressed, and used as stationary-phase probe sequence for hybridization testing of a biotinylated target sequence (Figure 1 a). The probe sequence was functionalized at its 5′ end with 4-ABA to provide oriented grafting.

Typical electroaddressing experiments are presented in Figure 2 for diazotate-free 4-ABA and the diazotated probe sequence. The diazonium reduction wave was observed at −1.1 V versus a carbon pseudoreference electrode with, in the case of the addressing of the modified probe, clear saturation of the electrode surface characterized by the decrease of the reducing current at −1.1 V versus C. The influence of the probe concentration (1–10 μM) during the electroaddressing step was studied. A peaklike profile was obtained and a maximum signal value was reached for SP biochips modified with 5 μM of probe. At higher probe surface densities, classical inhibitory effect[10] was observed owing to steric hindrance at the grafted surface. The probe surface density was estimated by an X-ray photoelectron spectroscopy (XPS) experiment (see the Supporting Information) and was found to be 3.75×10^{13} molecules cm^{-2}. This result compares well with similar XPS experiments for surface-coverage determination on gold substrates.[11] The spatial definition of the addressing was validated through the chemiluminescent image obtained after immobilization, hybridization, and detection. A typical

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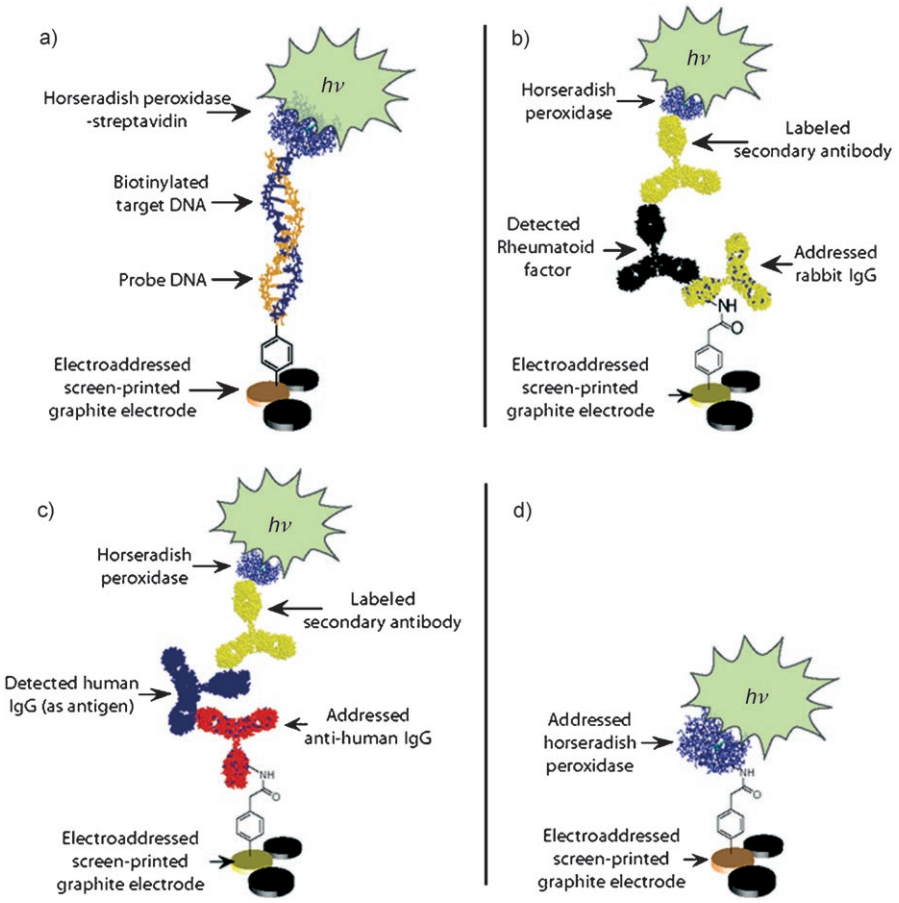

Figure 1. Different proofs of concept of the electroaddressed immobilization of a) a probe DNA sequence, b) an immunoglobulin antigen, c) an active anti-human antibody, and d) an active enzyme (horseradish peroxidase). The aniline-modified biomolecules are immobilized at the surface of 0.2-mm^2 screen-printed graphite electrodes.

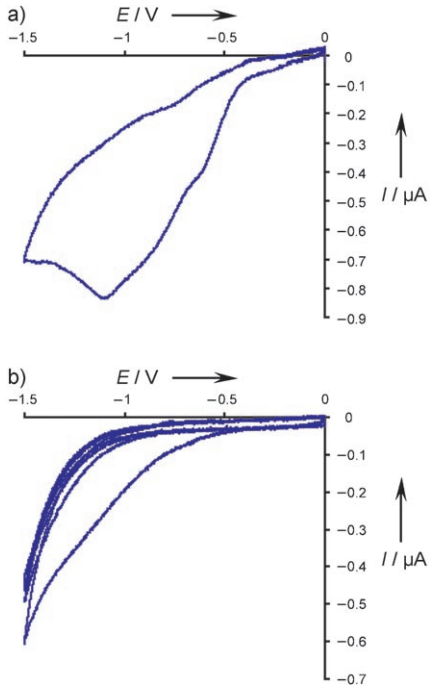

Figure 2. Cyclic voltammograms of a) 4-aminobenzylamine (4-ABA; 10 mM) and b) a 4-ABA-modified probe sequence (10 μM). Conditions: scan rate: 200 mVs^{-1}; 15 mM HCl, 15 mM $NaNO_2$.

micrograph obtained for the detection of a 25 nM target sequence is shown in Figure 3a. Three electrodes of the biochip are electroaddressed (nos. 1, 4, and 7) and produce a chemiluminescent signal, whereas no signal is obtained either from the nonaddressed electrodes or from the insulating layer and the plastic foil of the working area.

Similar studies were performed with 4-carboxyaniline (4-CMA) modified proteins as addressed biomolecules. First, rabbit immunoglobulins (IgG) were used as immobilized antigens and involved in the detection of rheumatoid factor (RF)—a family of human antibodies largely involved in rheumatoid diseases,[12] the presence of which could be detected by anti-rabbit-IgG activity of the serum. This assay, a capture format (Figure 1b), is not considered a sandwich assay because the immobilized rabbit IgGs are not used as active antibodies but as capture antigens. Nevertheless, the success of the assay is determined by the accessibility of the different epitopes of the immobilized IgG toward the numerous paratopes of the polyclonal human sera antibodies.[13] The immobilized protein layer is probably composed of both native and denaturated antibodies as a consequence of the necessary acidic conditions used during the diazotation step.

The chemiluminescent images obtained after capture of the RF (Figure 3b) again show the absence of signal from surfaces other than those of the addressed electrodes (nos. 2 and 7). The addressing of the antigen proteins was thus specifically localized on the chosen electrodes and no nonspecific adsorption of the different components of the assay occurred on the biochip surface.

Different human serum samples containing known concentrations of rheumatoid factor were incubated on the

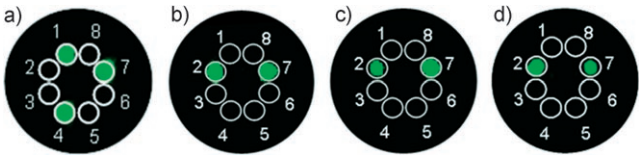

Figure 3. Chemiluminescent micrographs of a) a probe sequence electroaddressed (at electrode nos. 1, 4, and 7) biochip hybridized with target sequence 25 nM, b) a capture assay biochip incubated with RF 76 IU mL^{-1} (IU = international unit; addressed at electrode nos. 2 and 7), c) a sandwich assay biochip detecting 60 nM of human IgG (addressed at electrode nos. 2 and 7), and d) a horseradish peroxidase electroaddressed biochip (addressed at electrode nos. 2 and 7).

rabbit-IgG-modified biochip surface. A clear correlation between the measured chemiluminescent signal and the concentration of RF in the serum samples was found, which allows the detection of RF in the range 5.3–485 IU mL^{-1} with an acceptable accuracy comparable to those of previous works (standard Auraflex ELISA test and reference [13]).

For the immobilization technique to be fully demonstrated as useful for biosensing, a second immunochemical application based on a sandwich immunoassay procedure is presented. It involves the recognition properties of electroaddressed anti-human IgGs. In this case, the binding properties of the grafted antibodies are directly implicated in the detection process of the antigen in solution, in our case, a human IgG (Figure 1c). Thus, the loss of integrity of every immobilized antibody would have a dramatic effect on the recognition event. The chemiluminescent micrograph obtained after the completion of the sandwich assay (60 nM of free human IgG) on a anti-human-IgG-modified biochip shows clearly that the system is operational (Figure 3c). Indeed, no nonspecific interactions could be detected and the biochip provided very intense specific signals emitted only from the electroaddressed working electrodes (nos. 2 and 7). The immobilized anti-human-IgG antibodies were thus involved in the recognition of the free target protein, which shows that the proposed immobilization procedure maintains a convenient structural conformation of the grafted biomolecules, thus allowing them to be implicated in the recognition process.

Finally, a proof of concept for the immobilization of enzymes was performed with 4-CMA-modified horseradish peroxidase directly electroaddressed at the surface of a screen-printed graphite biochip. Because of the fragility of the biocatalyst, the diazotation conditions were modified (pH was increased to 2.5; see the Supporting Information). Under these conditions, the immobilized horseradish peroxidase was found to be fully active. The micrograph obtained (Figure 3d) shows an intense chemiluminescent signal localized at the electroaddressed electrodes (nos. 2 and 7). Such results are really promising in view of the interest of the biosensor community[14] in the immobilization of heme-containing enzymes on the surfaces of conducting materials and their direct electron transfer for sensing hydrogen peroxide. In this case, with a maximum distance of 1.5 nm between the enzyme and the electrode, the system should present interesting properties.

In summary, we have shown that it is possible to electroaddress fragile biomolecules at the surface of screen-printed graphite electrode biochips. The proposed method, based on the biomolecules modification with aryl diazonium prior to the addressing, was used to immobilize fully reactive oligonucleotides, antigens, antibodies, and enzymes. Work is in progress in our laboratory to use the present versatile immobilization technology to produce biochips suitable for application.

Received: December 11, 2006
Revised: February 21, 2007
Published online: April 13, 2007

Keywords: biochips · diazo compounds · electrochemistry · immobilization · proteins

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