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# Electropolymerization as a Versatile Route for Immobilizing Biological Species onto Surfaces

*Application to DNA Biochips*

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## Abstract

Biosensors based on electronic conducting polymers appear particularly well suited to the requirements of modern biological analysis—multiparametric assays, high information density, and miniaturization. We describe a new methodology for the preparation of addressed DNA matrices. The process includes an electrochemically directed copolymerization of pyrrole and oligonucleotides bearing on their 5' end a pyrrole moiety. The resulting polymer film deposited on the addressed electrode consists of pyrrole chains bearing covalently linked oligonucleotides (ODN). An oligonucleotide array was constructed on a silicon device bearing a matrix of 48 addressable 50 × 50 μm gold microelectrodes. This technology was successfully applied to the genotyping of hepatitis C virus in blood samples. Fluorescence detection results show good sensitivity and a high degree of spatial resolution. In addition, gravimetric studies carried out by the quartz crystal microbalance technique provide quantitative data on the amount of surface-immobilized species. In the case of ODN, it allows discrimination between hybridization and nonspecific adsorption. The need for versatile processes for the immobilization of biological species on surfaces led us to extend our methodology. A biotinylated surface was obtained by coelectropolymerization of pyrrole and biotin-pyrrole monomers. The efficiency for recognition (and consequently immobilization) of R-phycoerythrin-avidin was demonstrated by fluorescence detection. Copolymerization of decreasing ratios of pyrrole-biotin over pyrrole allowed us to obtain a decreasing scale of fluorescence.

**Index Entries:** Electrochemical polymerization; functionalized polypyrrole; DNA chip; biosensors; genotyping; biotin.

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## Introduction

There is a need for microanalysis devices that increase the number of analyzed parameters in a sample at a low cost, while decreasing the sample volume. To reach the high information density on a small surface necessary for this, it is essential to develop a versatile addressing system. Biosensors based on electronic conducting polymers (ECPs) *(1)* appear particularly well suited for these requirements of modern biological analysis. Indeed, the ECPs were attractive materials for the elaboration of a sensitive layer at the surface of an electrode. This is owing to their key properties: an easy electrodeposition on the electrode surface allowing miniaturization, and a versatile functionalization either by grafting or doping, enabling one to perform selective recognition.

Many biosubstances such as enzymes *(2)* or peptides *(3,4)* have been immobilized on an ECP matrix that is mainly a polypyrrole, PPy film. Indeed, the conditions of the electrodeposition of pyrrole monomers (low potential, buffered aqueous solution) were compatible with the stability of the biological entities.

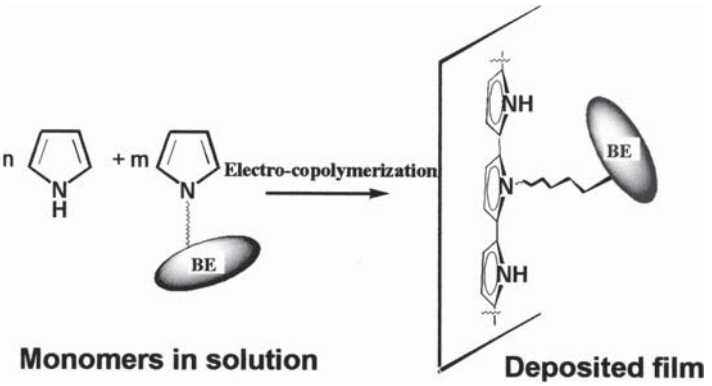
Thanks to the biocompatibility of the polypyrrole, we have recently shown that it has even been possible to include single-stranded DNA (ssDNA) in the bulk or at the surface of polypyrrole films. The immobilization of ssDNA can be realized either by copolymerization *(5)* (Scheme 1) or by postfunctionalization *(6)*.

The latter technique was based on the electrooxidation of a mixture of pyrrole and ODN-pyr (pyrrole covalently linked to an oligonucleotide via a spacer arm). The process leads in one step to the irreversible immobilization of ODN units (in a solid copolymer film formed by an oligonucleotide grafted onto a polypyrrole chain) on an electrode.

Copolymerization functionalization was extended to the grafting of peptides and was illustrated by immunodetection on a polypyrrole peptide chip *(7)*. More recently, we took up this concept for the biotin-avidin system. From the copolymerization of pyrrole monomers and a pyrrole linked covalently to a biotin entity through a spacer arm, we have synthesized *(8)* a polypyrrole film bearing biotin units, anchoring points of avidin conjugates via the strong affinity between avidin and biotin.

In addition to the versatility of functionalization, the copolymerization process allows the elaboration of microbiosensors *(9)*: an oligonucleotide array constructed on a silicon device bearing a matrix of addressable 50 × 50 μm gold microelectrodes. The DNA chip was prepared by successive electrochemical copolymerizations. This is achieved by successive electrical addressings of each gold microelectrode by selective switching with the resulting polymer growth being strictly limited to the selected electrode surface, and takes place on the activated electrode and not on its neighbor. Therefore, one can cover each gold dot by a polypyrrole grafted by a different ODN.

To illustrate the features presented by the coelectropolymerization process, we report herein two examples, with a particular emphasis on the




Scheme 1. Synthetic strategy for the immobilization of biological entities (BE) by copolymerization.

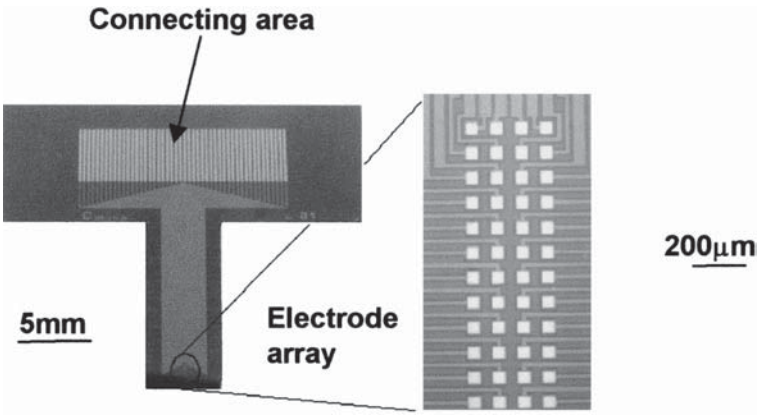


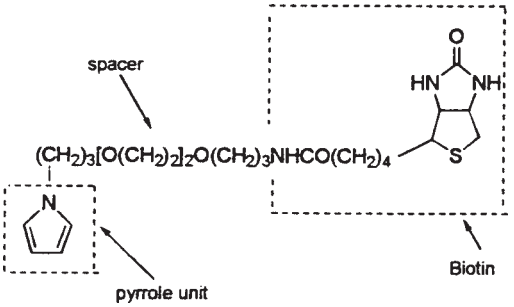

Fig. 1. Silicon chip and view of the array bearing 48 gold microelectrodes.

quartz crystal microbalance (QCM) technique, to follow the biomolecular recognition process. The first concerns the genotyping of hepatitis C virus in blood samples using a silicon chip bearing gold microelectrodes. The second concerns the preparation by copolymerization of polypyrrole layers functionalized with biotin units and carried out on microelectrodes arrayed on a silicon chip.

## Materials and Methods

### *Silicon Chips*

The matrix of 48 (4 × 12) gold microelectrodes (50 × 50 μm) was made by microelectronic technologies on a silicon support (CEA/Leti, France) as previously described *(9)*. Each electrode is individually connected (*see* Fig. 1). To avoid cross-polymerization between adjacent electrodes, a very high interelectrode insulation (>20 MΩ) was necessary. We found the silicon dioxide insulation layer compatible with DNA experiments.




Scheme 2. Biotinylated pyrrole **1**.

## *Preparation of Grafted Biomolecules*

ODNpyr were synthesized according to the previously described procedure by using a pyrrole-phosphoramidite building block in the course of the ODN synthesis *(5)*. Biotinylations of ODNs were carried out in the same way *(10)*.

The biotinylated pyrrole **1** (Scheme 2) is synthesized by coupling an amino-alkyl pyrrole and an activated ester of biotin *(8)*.

## *Synthesis of Polypyrrole Support*

Electrochemical syntheses and their characterizations by cyclic voltamperometry were made with a PAR 273 potentiostat from EG&G Princeton Applied Research controlled by a computer. All potentials are relative to a saturated calomel electrode (SCE). Electrochemical reactions were carried out in a 1-mL Teflon cell including a platinum wire as the counterelectrode and an SCE as a reference electrode. For oligonucleotides, the copolymerization reactions were carried out in 500 µL of a solution containing 20 m*M* pyrrole (Tokyo Kasei), 0.1 *M* $LiClO_4$ (Fluka), and 1 µ*M* ODN-pyr. The films were synthesized on the working microelectrode by cyclic voltamperometry (potential sweeping between –0.35 and +0.85 V vs SCE, scan rate of 100 mV/s). The reaction was stopped when the charge reached 250 nC. After each synthesis, the support and the cell were rinsed, another electrode was selected and switched on, and the next copolymerization was carried out (*see* Fig. 2).

For biotin immobilization, the electrocopolymerization conditions are the same as for oligonucleotides. Biotinylated pyrrole concentrations were from 100 to 0.01 m*M*.

## *Molecular Recognition and Detection of Bound Material*

The DNA chips were prepared with three (hepatitis C virus [HCV]) typing oligoprobes and a negative control (polypyrrole alone). These probes were individually copolymerized with pyrrole on the chip and were hybridized with clinical samples as follows (*see* Fig. 3). The HCV RNAs were extracted from known patient sera. They were reverse transcribed

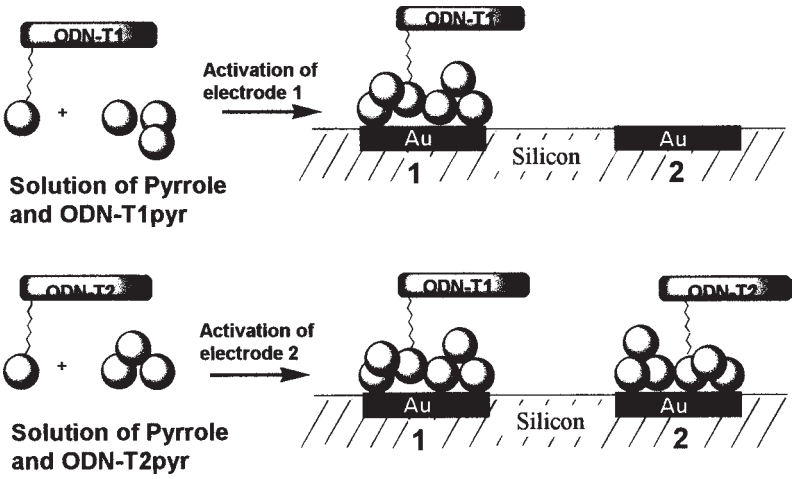



Fig. 2. Principle of the ODN immobilization by electrodirected copolymerization of pyrrole, and pyrrole grafted by ODN. ODN-T1pyr is first copolymerized on the electrode 1; following washing of the cell, copolymerization of the ODN-T2pyr is carried out by activation of electrode 2, and so on.

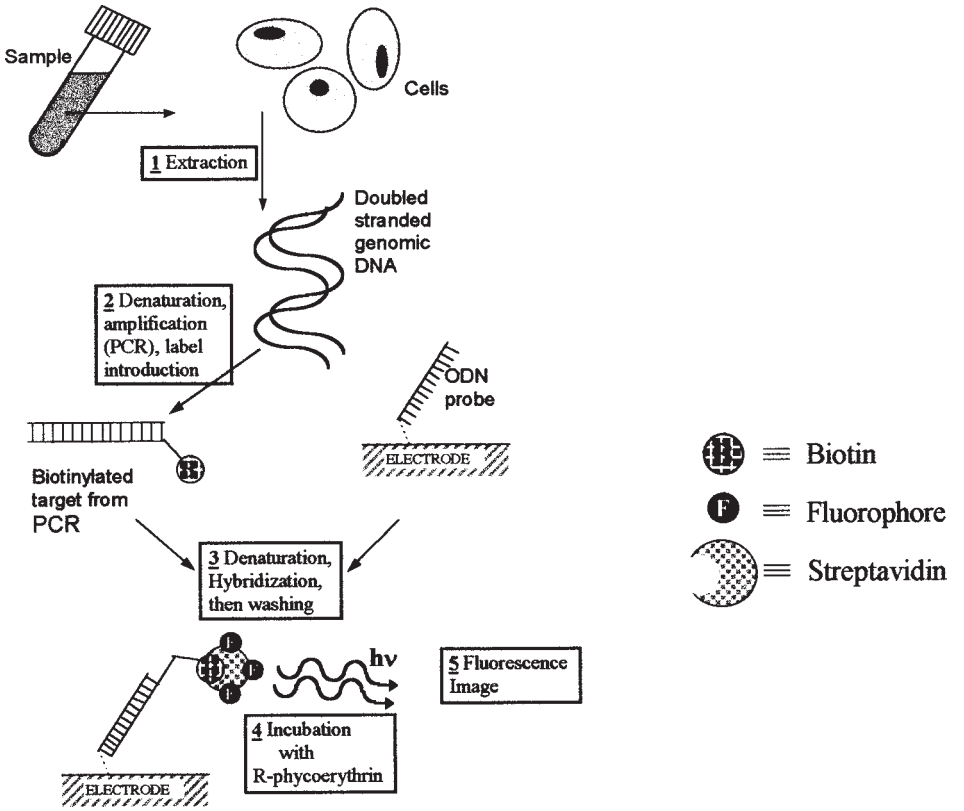



Fig. 3. Principle of DNA detection from clinical samples.

and amplified by a polymerase chain reaction (PCR) reaction in the presence of a sense biotinylated primer. The obtained biotinylated fragments were hybridized to the probes immobilized on the microelectrodes. The genotyping was realized by successive hybridizations on the DNA chip. After each hybridization, the chip was incubated for 10 min in a buffered solution of 5% streptavidin-R-phycoerythrin, then rinsed, and, finally, the fluorescence was recorded for 1 s with an epifluorescence microscope (BX 60, Olympus) equipped with a Peltier cooled charge-coupled device camera (Hamamatsu) and image analysis software. Each detection was followed by a 1-min denaturation step in 0.1 *M* NaOH in order to regenerate the chip.

The biotin detection was carried out in a similar way. The biotinylated chip was incubated for 10 min in a buffered solution of 5% streptavidin-R-phycoerythrin. However, to avoid any side immobilization of the streptavidin-R-phycoerythrin by nonspecific adsorption on polypyrrole, the biotinylated support was previously blocked by washing with Denhardt's reagent.

### *Gravimetric Studies*

The quartz crystal was AT-cut (manufactured by International Crystal Manufactoring Company Inc., Oklahoma City, distributed by EG&G) with a basic resonance frequency of 8.8 MHz and sandwiched between two Pt electrodes (5 mm diameter). The frequencies were measured by an HP 53424 A frequency counter from Hewlett Packard.

The deposition of ODN on the QCM electrode was carried out in an aqueous solution with 0.1 *M* $LiClO_4$ containing 20 m*M* pyrrole and 12 μ*M* ODN-pyr. The films were synthesized by cyclic voltamperometry (potential sweeping between –0.6 and +0.85 V vs SCE, scan rate of 50 mV/s). The reaction was stopped when the charge reached 100 mC/cm. After thorough rinsing, the film was held at 0 V/SCE during the QCM experiment to minimize ionic exchanges with the PPy layer and DNA nonspecific adsorption.

The deposition of the biotinylated copolypyrrole (noted copoly[**1**]) on the QCM electrode was carried out in an aqueous solution containing 3% $CH_3CN$ with 0.1 *M* $LiClO_4$ (Fluka Purum) containing 20 m*M* pyrrole and 10 m*M* biotinylated pyrrole **1** that was purged by argon bubbling before each experiment. The electrosynthesis of the copoly[**1**]) was realized by repeated potential linear scanning between –0.35 and +0.85 V.

## Results and Discussion

### *HCV Genotyping on DNA Chips*

Before undertaking the HCV genotyping procedure, optimizations of the support preparation were carried out using the ODN-G probe. Film thickness (evaluated through the charge passed for the synthesis) was varied from 10 to 30 nm for copoly(pyrrole/ODN-Gpyr) film. After a fast increase, the fluorescence reached a plateau for a thickness of 15 nm. The

optimization of the film thickness (250 nC, 20 nm) led to a rapid synthesis of a homogeneous polymer layer and to a perfect covering of the gold microelectrode.

The ratio between pyrrole and ODN bearing a pyrrole group (ODN-pyr) is of major importance. The concentration of pyrrole for the electropolymerization in aqueous media cannot be decreased below 20 m*M*. Conversely, only the concentration in ODN-Gpyr has been modulated from 0.12 to 3 m*M*. Results of fluorescence analysis show that a saturation of the polypyrrole surface occurred when the concentration in ODN-Gpyr reached 0.6 m*M*.

The specificity of the affinity between the ODN probe and the target HCV was studied by QCM, one side of the quartz Pt electrodes being covered with a film of copoly(pyrrole/ODN-T1pyr) *(11)*. The exposure of this modified side to chemically synthesized 20-base sequences, either the complementary ODN, HCV T1, or the noncomplementary HCV T2, was interrupted by denaturation, irreversible dehybridization, by soda (0.2 *M*, 20 s), allowing us to continue with another reactant. Simple PPy was also exposed to the same treatment as the blank experiment.

Figure 4 shows that a drift owing to ionic exchanges was observed on both films, but only the copolymer reacted toward the hybridization. The total amount of adsorbed HCV T1 was 48 ng. By comparison, the nonspecfically adsorbed HCV T2 was 7 ng. Consequently, assuming the nonspecific adsorption of HCV T1 is almost the same as the nonspecific adsorption of HCV T2, the hybridization involved 41 ng, corresponding to 205 ng/cm$^2$. This value is in agreement with those reported by Nicolini et al. *(12)* determined by QCM on LB film consisting of ssDNA sandwiched between two monolayers of octadecylamine. The weak nonspecific adsorption of ODN either on the copolymer or simple PPy is also in agreement with the value reported by Saoudi et al. *(13)* on the adsorption of DNA on polypyrrole powder. In conclusion, the use of these selected conditions (buffered medium, reduced PPy; *see* Materials and Methods) results in a very low nonspecific adsorption of DNA.

The specificity of the DNA target recognition was also confirmed by using fluorescence detection and by performing hybridization using a specifically synthesized biotinylated complementary ODN-G under saturating conditions (250 p*M* in the hybridization buffer). No cross-contamination with ODN-T1pyr or ODN-T2pyr was evidenced.

The sensitivity level was evaluated by end point dilutions of the same complementary ODN-G. A high fluorescence signal was observed for concentrations >$10^{-11}$ *M*; however, it decreased for $10^{-12}$ *M* ($12 \times 10^6$ copies in 20 μL) and was negative for $10^{-13}$ *M*. For the electrode area, this corresponds to 4800 mol/mm$^2$.

To increase the hybridization signal for long DNA fragments, the efficiency of a 5'-end polythymidine arm was evaluated. HCV probes bearing 3, 5, or 10 thymidines at their 5' end were evaluated. Results showed that the fluorescence was higher with five thymidines or more. Pentathymidine arms (5' end) were further used for all pyrrole HCV probes.

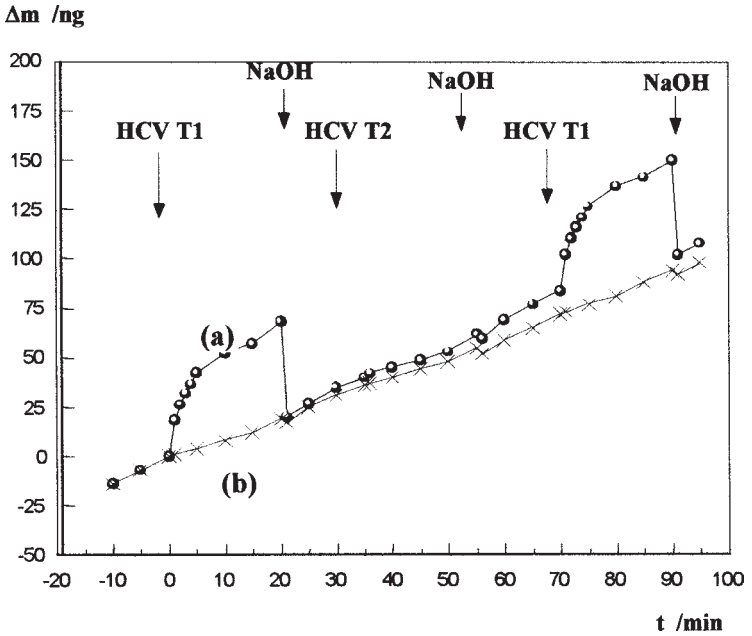



Fig. 4. Evolution of the electrode inertial mass vs time on film of (a) copoly(pyrrole/ODN-T1pyr), and (b) simple polypyrrole. The arrows indicate the successive injections of the complementary HCV T1 or the noncomplementary HCV T2.

For HCV genotyping of clinical samples, the DNA chips were prepared according to the procedure described at the beginning of the Synthesis of Polypyrrole Support section with the three HCV typing oligoprobes (i.e., ODN-Gpyr, ODN-T1pyr, and ODN-T2pyr) and a negative control (polypyrrole alone). These probes were copolymerized on the areas of the chip according to the pattern shown in Fig. 5A. Figure 5B shows the fluorescence results obtained with type 2 RNA: hybridization of G and T2 probes. Therefore, an easy determination of HCV genotype 2 was obtained without any ambiguity and was in accordance with the results obtained by other methodologies. The DNA chips can be stored for at least 3 mo without loss of activity under moist conditions at 4°C.

### *Biotinylated Polypyrrole, Copoly[**1**]*

In accordance with the copolymerization functionalization, copoly[**1**] films were electrosynthesized from pyrrole and the biotinylated pyrrole **1** (Scheme 2) on microelectrodes. The biotinylated pyrrole used was a biotin entity linked to the nitrogen atom of a pyrrole unit by a hydrophilic arm (alkoxyl chain composed of 3 oxygen and 10 methylene groups). The hydrophobic/hydrophilic balance as well as the length of the spacer arm were selected to allow good solubility and accessibility of the hanging biotin *(8)*. The accessibility of immobilized biotin entities toward avidin conjugates has been demonstrated by QCM experiments *(14)*.

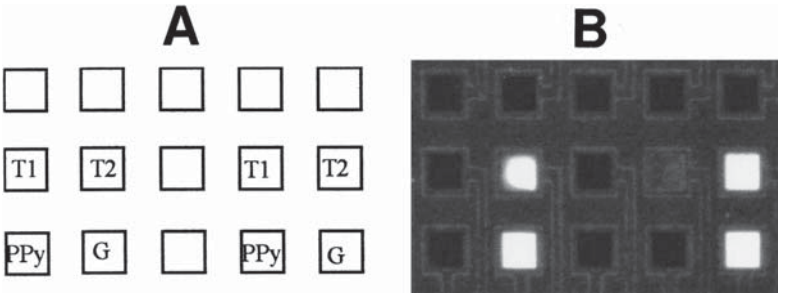



Fig. 5. **(A)** Pattern of probe repartition on the microelectrode array: PPy, polypyrrole homopolymer; G, T1, T2, polypyrrole copolymers bearing probes ODN-Gpyr, ODN-T1pyr, and ODN-T2pyr, respectively. **(B)** Fluorescence result of genotyping of a clinical sample positive for HCV T2 on the array described in (A).

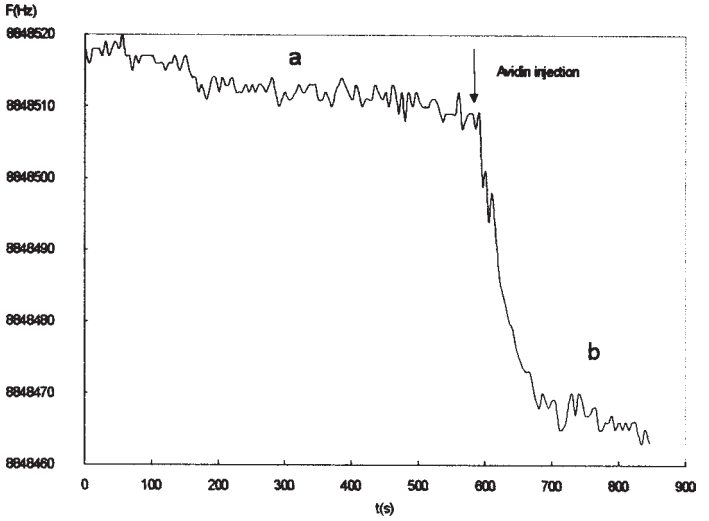



Fig. 6. Variation of frequency with the time of the copoly[**1**] film in aqueous solution containing the Denhardt's reagent, before (a) and after (b) the injection of an avidin solution.

After the electrosynthesis on the platinum surface of the QCM, the copoly[**1**] film was placed in an aqueous solution containing Denhardt's reagent in order to avoid nonspecific adsorption of avidin. The response of QCM (Fig. 6) before and after the injection of an avidin solution shows a fast decrease in frequency just after the addition of avidin conjugate. From this result, we have assessed that the mass of avidin immobilized on the copoly[**1**] film is equal to 48 ng, corresponding to 0.7 pmol of avidin conjugates immobilized on the copoly[**1**] film.

The same experiment was conducted with a simple polypyrrole film. In this case, the injection of avidin did not induce a significant variation in the frequency in comparison with the one observed for the biotinylated copolypyrrole.

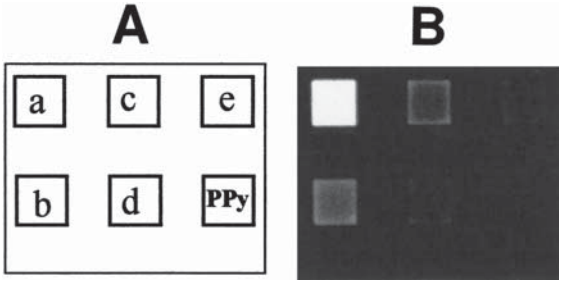



Fig. 7. **(A)** Pattern of the biotin repartition on the microelectrode array. Electrosyntheses were carried out from 20 m*M* pyrrole and a decreasing range of biotin pyrrole with a synthesis charge of 18 mC/cm² (about 60 nm thickness). PPy, polypyrrole homopolymer; a, b, c, d, e, copoly[**1**] synthesized in presence of 100, 10, 1, 0.1, and 0.01 μ*M* biotinylated pyrrole **1**, respectively. Each electropolymerization was followed by a thorough washing step and a blocking step. **(B)** Fluorescence results after revelation process using a solution of streptavidin-R phycoerythrin followed by washing in a phosphate buffer.

Using various concentrations of biotinylated pyrrole for the same concentration of pyrrole monomers, we obtained different copolymers containing variable amounts of biotin. Figure 7A shows the pattern of the deposition of these areas. The presence of biotin was analyzed from a fluorescence measurement owing to streptavidin bearing a fluorescent phycobiliprotein. The anchoring of the streptavidin to the immobilized biotin was easy via the strong biotin/avidin affinity. Figure 7B clearly shows that the intensity of the fluorescence is related to the amount of biotin involved in the copolymerization reaction and yields the amount of accessible biotin at the surface of the film.

## Conclusion

This technology appears to be versatile and very precise with routine synthesis on 50-μm electrodes. There is no need for specific instrumentation for the manufacturing of the biochip (copolymerization), in contrast with other methodologies involving photochemistry or robotic deposition.

The electrochemical addressing concept requires the synthesis of tailor-made monomers and implies several electrochemical deposition experiments equal to the number of modified microelectrodes. However, it results in a perfect control of the purity of the immobilized DNA sequence (contrary to *in situ* synthesis of ODN) and is perfectly adapted for targeted genotyping.

The immobilization of biotin opens the route to the elaboration of many biosensors owing to the commercial availability of a wide variety of avidin conjugates of biomolecules. In addition, the fluorescence scale demonstrates the feasibility of the quantification of the titration.

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